OBSERVATIONS ON THE DIAGNOSIS OF BOVINE VIRUS DIARRHOEA IN THE LIVE ANIMAL

By

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ABSTRACT

Confirmation of a provisional diagnosis of bovine virus diarrhoea in the live animal is difficult. The best method proved to be the isolation of virus in cell cultures from nasal and faecal samples, virus being sought after three blind passages either by its ability to interfere with a cytopathic strain of the virus or by identifying specific antigen by immunofluorescence.

Reliability was only achieved when strict controls are applied at each stage; in particular cell cultures and sera had to be shown to be free of virus and/or antibody. For example 40 percent of the bovine embryonic kidneys collected for the preparation of cell cultures were found to be infected and most lots of foetal calf serum and all new born calf sera contained antibody.

Cryopreservation of bovine embryonic kidney and calf testes cells with Dimethyl sulphoxide did not affect their viability or susceptibility to bovine virus diarrhoea virus while aliquots were screened for viruses. More samples gave virus in lamb testes cells, but the lamb testes cells deteriorated on storage. Further improvements were achieved when cells were propagated in antibody free serum which was inactivated at a temperature high enough to inactivate the virus without affecting the growth promoting activity of the serum and maintained in serum-free medium.

The relationship of bovine virus diarrhoea virus and rubella virus was investigated. Rubella virus interfered

with bovine virus diarrhoea virus in bovine embryonic kidney cells but there was no cross neutralization between them and bovine virus diarrhoea antibodies did not inhibit haemagglutination by rubella virus.

All strains of bovine virus diarrhoea virus and border disease virus were so closely related that they were considered to be strains of the same virus.

This Thesis was composed by me and describes the original work I carried out under the supervision of Professor Sir Alexander Robertson and Dr. G.R. Scott.

LIST OF ABBREVIATIONS

AGD = Agar gel diffusion

BD = Border disease

BDM = Border disease virus, Moredun strain.

BDV = " " "

BDW = " " " Weybridge strain.

BEK = Bovine embryonic kidney.

BVD = Bovine virus diarrhoea.

BVD-BEK = " " " virus propagated in BEK.

BVD-LT = " " " " " " lamb testes cells.

CF = Complement fixing.

CP = Cytopathic.

CPE = Cytopathic effect.

CT = Calf testes.

DDW = Deionized distilled water.

DMSO = Dimethyl sulphoxide.

EBSS = Earle's balanced salt solution.

EYL = EBSS + yeast extract + lactalbumin hydrolysate.

FCS = Foetal calf serum.

HBSS = Hank's balanced salt solution.

HC = Hog cholera.

HCV = Hog cholera virus.

LAH = Lactalbumin hydrolysate.

LEBSS = Lactalbumin hydrolysate in Earle's balanced salt solution.

LHBSS = Lactalbumin hydrolysate in Hank's balanced salt solution.

IFT = Indirect fluorescence technique.

IT = Interference test.

LS = New born lamb serum.

LT = Lamb testes.

MEME = Minimum essential medium in Earle's balanced salt solution.

MEMH = Minimum essential medium in Hank's balanced salt solution.

NADL = National Animal Disease Laboratory strain of BVDV.

NBCS = New born calf serum.

NCP = Noncytopathic.

NEA = Non - essential amino-acids.

PBS = Phosphate buffered saline.

RP = Rinderpest.

RPV = Rinderpest virus.

RS = Rabbit serum.

RV = Rubella virus.

SNT = Serum neutralization test.

SS = Adult sheep serum.

STV = Saline-trypsine-versene

TPB = Tryptose phosphate broth.

YE = Yeast extract.

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Rinderpest and bovine virus diarrhoea, Cell culture hazards, Serum hazards, Alleviation of hazards, Production of monospecific sera, Subclinical bovine virus diarrhoea infection, Bovine virus diarrhoea and rubella viruses, Bovine virus diarrhoea and border disease viruses.

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GENERAL INTRODUCTION

The realisation of the worldwide distribution of bovine virus diarrhoea (BVD) quickly followed the description of the disease by Olafson and his colleagues (1946). In spite of the involvement of the disease in abortion and its severity in young animals (Kahrs, 1971) it is considered as a benign condition (Van Bekkum and Straver, 1966). Its importance lies in its alarming simulation of serious conditions like foot and mouth disease and rinderpest.

In the past sheep were incriminated in the dissemination of bovine virus diarrhoea (Bögel, 1966), an interesting allegation in the light of the discovery of a serologically related virus affecting sheep, namely border disease virus (BDV) which has been shown to be infective for the bovine foetus.

The diagnosis of BVD is complex because of the ubiquity of the virus. For example, Borgen (1963a) found serum neutralizing antibodies in 91 percent of the animals he tested. Schell et al., (1972) found that more than 30 percent of the bovine kidneys that they were using for cell cultures were infected with BVDV. Smithies and Modderman (1975) tested commercial foetal calf sera and reported that 75 percent were contaminated with noncytopathic (NCP) strains of the virus. The NCP strains of BVDV are more prevalent (Gillespie, 1968) and complicated tests are needed for their identification.

The purpose of this study was to identify the best method or methods for confirming a diagnosis of BVD in the live animal.

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INTRODUCTION

The emergence of a new disease of cattle was reported in 1946 by Dr. Olafson and his colleagues in New York State, U.S.A. and independently by Dr. Childs in Saskatchewan, Canada. Dr. Olafson's group recognised that the new disease was highly contagious, caused by a virus and characterized by fever, diarrhoea, leucopaenia, rhinorrhoea, depression, anorexia, dehydration and abortion in some cases.

The worldwide distribution of the disease was delineated by reports from Europe (Hedstorm and Isaksson, 1951), Africa (Otte and Peck, 1960), Asia (Pande and Krishnamurthy, 1961) and Australasia (Salisbury et al., 1961). In fact the disease was found wherever it was looked for; the only exception was that reported by Blacou and colleagues (1975) who tested 273 bovine sera in Malagasay and found them negative in the serum neutralization test. They were also unable to isolate the virus from samples collected from suspect animals.

AETIOLOGY OF THE DISEASE

Olafson et al. (1946) concluded that the disease was caused by a virus, hence the name "viral diarrhoea" (VD). Later Lee and Gillespie (1957) were able to propagate the New York -1 (NY-1) strain of the virus (Baker et al., 1954) in cell cultures.

Strain variations were observed by Pritchard and his colleagues (1956) when they compared the Indiana-46 (Ind.46) with NY-1 and reported that it was immunologically different. This led to the establishment of another label for the disease namely, "mucosal disease" (MD). Gillespie et al., (1961), Kniazeff et al., (1961), Thompson and Savan (1963), Taylor et al., (1963) and Tyler and Ramsey (1965) studied different strains isolated from cases reported as being viral diarrhoea, and cases reported as being mucosal disease and found similarities among the different strains. Accordingly, the Committee on Terminology (1968) recommended that the condition be called bovine viral diarrhoea-mucosal disease (BVD-MD).

Fernelius et al., (1971) suggested three serotypes for the virus but Corthier and Aynaud (1973) reported that the differences among the strains did not warrant serotypes.

CHARACTERIZATION OF THE VIRUS

The virus was characterized as a small particle which was less than 50nm (Hermodsson and Dinter, 1962), enveloped and contained ribonucleic acid (RNA) (Hafez et al., 1968 and Niess, 1973). It was therefore suggested that the virus closely resembled arboviruses and hence could be grouped with

rubella virus (RV) and hog cholera virus (HCV) in a seperate subdivision of the Togaviruses (Diderholm et al., 1973; Richie and Fernelius 1969).

TRANSMISSION OF THE DISEASE

Olafson et al., (1946) wrote, "This malady can be transmitted at will and unless extreme care is used, can be carried from farm to farm". They infected susceptible animals by drenching them with infected faecal material or with tissue emulsion from febrile animals. In contrast Ramsey and Chivers (1953), Swope and Luedke (1956) and Olson and Hoerlein (1956), failed to produce the clinical picture of the disease by artificial inoculation or through contact. This led to the two different names of the disease; an easily transmissible, highly contagious and less fatal condition known as VD and a non-transmissible, less contagious and highly fatal condition labelled MD.

Bögel and Voss (1964) reported that animals chronically infected and having persistent viraemia constituted a hazard in addition to the subclinical cases. On the other hand, Ward and his colleagues (1969), observed that virus was not shed by experimentally infected and subsequently immune cows or by their offspring during the post-calving period.

Bögel (1964, 1966) postulated that, as in malignant catarrh, flocks of sheep should be considered as a possible reservoir of MD virus, a hypothesis that predated the recognition of a relationship between the viruses of bovine virus diarrhoea (BVDV) and border disease (BD). French et al.,

(1974) demonstrated transmission to sheep from infected cattle and contaminated food and Plant et al., (1976) suggested that BD of sheep was in fact a form of BVD. They reported a high content of virus in ovine foetal membranes at parturition suggesting the possibility of virus amplification during the lambing season.

The involvement of pigs in the transmission of the disease was also suspected by Fernelius and his colleagues (1973).

DISTRIBUTION OF THE VIRUS IN ANIMAL TISSUES

Olafson and his colleagues (1946) transmitted BVD with whole unclotted blood, faeces and splenic emulsion. Later, Huck (1957) transmitted the disease with citrated whole blood, plasma, lymph nodes and brain tissue. Gutekunst and Malmquist (1964) isolated the virus from buffy coats although the animals were showing appreciable titres of neutralizing antibody. In naturally infected animals, viraemia persisted throughout the febrile period disappearing in one to two weeks, but in severely affected animals which eventually died, viraemia persisted for three weeks (Saurat et al., 1973).

Bürki (1965) observed that the greatest amount of virus was in the mucous membranes of the head and intestinal tract, then in the tissues of the middle and lower respiratory tracts, and least in homogenates of liver, spleen and kidney tissues. Darbyshire and Huck (1966) recovered the virus from lungs, duodenal tissue and mediastinal lymph nodes of experimentally infected animals and Gillespie and colleagues (1967) isolated

the virus from the lungs, intestines, livers and spleens of aborted foetuses. Braun et al., (1973) recovered the virus from the brain, lungs, spleen, placenta, vitreous humour and allantoic and amniotic fluids.

Fernelius and Lambart (1969) examined tissues by virus isolation and antigen detection. The tissues most often positive for virus isolation and fluorescent antibody technique (FAT) were rectum, spleen, lungs and submaxillary lymph nodes and the tissues that contained cell culture infectious particles but were negative to the FAT were; hearts, kidneys and intestines. All heart tissues were negative in the FAT and lymph nodes, other than the submaxillary, had a relatively high percentage of fluorescence but seldom contained infectious virus. Using the FAT alone Meyling (1970) detected specific BVDV antigen in lips, tongue, oesophagus, small intestines, spleens, livers, kidneys, parotid glands, nasal mucosae, lungs, brains and corneas.

French et al., (1974) and Snowdon et al., (1975) inoculated pregnant sheep with different strains of BVD virus. They isolated the virus from the blood, kidneys, thymus, lymph nodes, and uterine caruncles of the ewes and kidneys, livers, spleens, membranes and fluids of foetuses. The highest titre was found in the placentomes.

DIAGNOSIS OF THE DISEASE

INTRODUCTION:

Clinicially it is extremely difficult and in most cases impossible to make a diagnosis of BVD because the consequences of infection diversely vary from one instance to

the other. Clinical and pathological syndromes as described in the literature do not provide satisfactory distinguishing criteria. They range from inapparent infection to severe fatal catarrh sometimes complicated by respiratory involvement and other infections. Chronic persistent cases are often misdiagnosed as other debilitating conditions.

Diseases which should be differentiated include malignant catarrhal fever, rinderpest, vesicular diseases, papular stomatitis, bluetongue, shipping fever and ingestion of caustic substances (Kahrs, 1971). Chronic cases have to be differentiated from chronic parasitism, the maladsorption syndrome, Johne's diseases and lymphosarcoma (Hull, 1972). Accordingly, clinical findings based on history, signs and lesions should be supplemented with necroposy findings, virus isolation and identification and testing of paired sera.

Serological confirmation of a diagnosis is surrounded with some difficulties. Chronic cases showing high levels of neutralizing antibody from which virus could be isolated present an impediment for diagnosis (Gutekunst and Malmquist 1964). Also inapparent infections resulting in high titres of antibody and chronic infections with no antibody responses pose great difficulties (Bürki and German, 1964).

Isolation and identification of BVDV is not a straightforward job. In particular, the presence of BVDV as an adventitious contaminant of bovine tissues and bovine sera including foetuses (Kniazeff, 1968, Kniazeff et al., 1967) complicate the issue.

Consequently many different methods have been designed for confirming a BVDV infection. These include a calf protection test, a variety of complex methods for isolation and identification of the virus, antigen detection and the demonstration of antibody.

CALF PROTECTION TEST:

Baker et al., (1954) maintained their NY-1 strain of BVDV as splenic material from an infected calf and kept it in dry ice with occasional transfer by inoculating susceptible calves. This strain served as a reference strain with which later isolates were compared in a calf cross-protection test. The drawbacks of this test were that it was expensive, slow, very difficult to get specific pathogen free calves, experimental infection might be very mild in which case one had to resort to unreliable tests such as leukocytic counts and finally resistence to infection could well be broken by a heavy challenge dose.

VIRUS ISOLATION IN TISSUE CULTURE:

Lee and Gillespie (1957) successfully propagated NY-1 strain of BVDV in bovine skin muscle and BEK cells. The strain did not produce CPE but continued to be infective for susceptible calves. Because of the presence of cytopathic (CP) and noncytopathic (NCP) strains of BVDV and the contamination of BEK cells and sera with the virus different workers have tried different media for growing BEK cells and many workers have tried different cell cultures for propagating the virus.

Cytopathic Strains (CP):

Underdahl et al., (1957) isolated a CP strain of BVDV in kidney cell cultures prepared from foetuses 12-15 in. long. Their growth media consisted of Hanks' balanced salt solution (HBSS), lactalbumin hydrolysate(LAH) and 20 percent of adult bovine serum. They thought that their serum for maintenance was antibody free because they prepared it from a newborn calf which had not suckled; intrauterine infection was not then known to occur. The cytopathic effect (CPE) started as small vacuoles on the 7th to 10th day post-inoculation. The vacuoles increased in size until the cells disintegrated on the 10th to 14th day post-infection. This strain was isolated from pooled tissue homogenates from outbreaks reported by Olson and Hoerlein (1956).

Noice and Schipper (1959) isolated a CP strain in BEK cell cultures. No serum was used for cell growth or maintenance, instead they used a mixture of 199, Morgan, Morton and Parker media. Van Bekkum (1959) isolated a CP strain in bovine foetal skin epithelium.

Gillespie et al., (1960) isolated a CP strain (Oregon C24V) and showed that it was immunologically homologous with the reference strain, NY-1. Their strain was isolated from a spleen emulsion which was prepared as a 10 percent suspension in Parker 199 medium and 0.2ml of the suspension inoculated into each tube containing a monolayer of BEK cells. CPE started on the sixth day post-inoculation as rounding and detaching of affected cells. Complete detachment occurred by the 12th to 15th day. In later passages CPE appeared within 2-3 days.

Preparations fixed in Bouin's fluid and stained with haematoxylin — eosin or Giemsa stains showed numerous cells with round intracytoplasmic inclusions, pyknotic and eccentric nuclei and excess of cytoplasmic vacuoles.

Antibodies produced in calves by both NY-1 and Oregon C24V cross neutralized. Kniazeff et al.,(1961) used this strain to detect the possible immunological relationship among the various strains of BVDV isolated in different parts of the world.

Gillespie et al., (1961) recommended for the routine isolation of CP strains, monolayers of BEK cells obtained from the cortices of 2-4 months bovine foetuses. The medium for growth was composed of HBSS and LAH and calf or lamb serum. Earle's balanced salt solution (EBSS) was used in maintenance medium. Gratzek (1961) however used both BEK and bovine testicular cells (BTC) and reported that BTC appeared to be admirably suited for BVD virus cultivation and cell associated virus could be demonstrated 8-10 hours and free virus 12 hours after inoculation.

Mills and Luginbuhl (1965) reported that the third passage of calf kidney cells appeared to give more consistent results. They used HBSS supplemented with LAH and 5 percent tryptose phosphate broth for maintenance of cell monolayers. The CPE was observed on day 5 in the tertiary cells as linear thinning of cells and pyknosis of nuclei. This was not observed in primary and secondary cells. Degenerative changes by the 7th day in the controls of primary and secondary

cell cultures simulated the CPE in infected cells. Primary bovine kidney cell cultures were difficult to maintain long enough without serum for virus neutralization to be read with confidence.

In a brief report Malmquist (1968) summarized the problems of tissue culture thus; although most frequently used the monolayers of bovine kidney cells were often not as sensitive or durable as bovine testicle cells. A cell line originating from bovine embryonic spleen was found to be more sensitive and quicker to indicate CPE than kidney cells. Also a cell line originating from embryonic bovine trachea (EBTr) and available through the "American Type Culture Collection" was found to be susceptible to BVDV.

Gutekunst and Malmquist (1964) isolated the virus in bovine buffy coats. Malmquist et al., (1965) adapted the CP National Animal Disease Laboratory (NADL) strain of BVD virus to a pig kidney cell line (PK-15). Marcus and Moll (1968) adapted also a virus strain to the Madin-Darby bovine kidney cell line (MD-BK). CPE appeared in 3-5 days as rounding and granulation of cells in scattered areas. This was followed by death and detachment of cells leaving empty spaces. Complete destruction and detachment of all cells usually occurred by the seventh day post-inoculation.

Fernelius, et al., (1969) tried to adapt the NADL and the C24V strains to cell lines such as hamster kidney (HaK) and a human cell line (ERK-1) but no CPE was produced. They also failed to produce CPE in PK-15 and the cytopathogenic particles for BEK were lost after the sixth passage in PK-15. They detected the infection by immunofluorescence .

Philip (1973) likewise failed to propagate BVDV in PK-15.

Scott et al., (1972) isolated a CP strain of BVDV from a bovine foetus in bovine foetal spleen (BFS), Bovine foetal lung (BFL) and BEK cell cultures. In BFS cells vacuolations began to appear by the second post-inoculation day and progressed to involve all the cells by the third day. Vacuolation was the most consistent feature. By the sixth day only cell fragments remained. In BEK cells, cell destruction and stranding occurred with a high concentration of virus inoculum. Vacuolations were not prevalent. Marked elongation and stranding of infected cells was evident with some cells extending across a 500X field. Pyknotic cells were observed. In BFL cells, over 50 percent of the cells infected with the undiluted virus contained cytoplasmic vacuoles, ten to more than 50 vacuoles per cell. Cells remained epithelial with little stranding. In all, the CPE in BFL was the most difficult to detect.

Philip (1973) isolated two CP strains which replicated very well in primary calf kidney cells grown in medium containing bovine serum. The same strains showed less growth in bovine lung and MD-BK cell line. Archbald et al., (1973) propagated a CP BVDV strain in bovine endocardial cells.

McClurkin et al., (1974) reported the propagation of the BVDV in low and high passage of bovine turbinate cells prepared from a colostrum-deprived calf removed surgically from a seronegative dam. CPE started as vacuolations of cells on the third to fifth day, with complete destruction of the cell

sheet within five to six days. Cancellotti and Turilli (1975) reported the susceptibility of the bovine embryonic kidney cell line (AU-BEK) established by Rossi and Kiesel (1973) to BVDV.

Non-cytopathic Strains (NCP)

NY-1 strain did not produce CPE in cell cultures (Lee and Gillespie, 1957); the authors detected the presence of the virus in culture by infecting susceptible calves with the 20th passage and the 35th passage. They propagated their BEK cells in EBSS supplemented with LAH and ten percent inactivated horse serum and used one percent horse serum for maintaining the cultures. Claflin et al., (1959) propagated another NCP strain in BEK and referred to it as Indiana-46 (IND-46). This strain was noncytopathogenic up to the 26th passage in cell culture (Gillespie and Baker, 1959).

In early work, calf inoculation was the only indicator for the presence of NCP strains in cell cultures. Later, simpler and easier tests were developed, namely :

Interference test and plaque reduction technique : A plaque inhibition assay for NCP strains of BVDV was described by Gillespie, et al., (1962). Cells from BEK representing the second through the sixth passage were grown in medium consisting of EBSS supplemented with 0.5 percent LAH and 10 percent lamb or calf serum. The workers reported that of the CP strains, C60F produced the most clearly defined plaques and so it was chosen as the indicator virus. When cell monolayers were inoculated with an NCP strain three days prior to inoculation with the C60F, they became resistant and so fewer plaques were formed. If the NCP strain was

neutralized by a homologous antiserum before being inoculated in cells, the normal number of plaques were formed by the cytopathic strain. This method made possible the titration and quantitation of NCP strains of BVDV.

A plaque assay system for the BVD-Oregon C24V strain has also been developed (Gratzek et al., 1967). It was composed of bovine testicular cell monolayers overlaid with Hank's Balanced Salt solution, lactalbumin hydrolysate, Tris buffer, horse serum and agar. Plaques were seen after 72 hours of incubation at 36°C. They were approximately 2mm in diameter. Plaques were also formed by the NADL strain of BVDV on BEK, CT bovine lung cells and on lamb testicle cells (Singh, 1969). Straver (1971) claimed that NCP strains formed plaques when the plates were left in the dark for several days after staining.

Enhanced Newcastle disease (END) test: This test was developed in Japan for the detection and identification of NCP strains of hog cholera virus (HCV) and Inaba et al., (1963) applied it for the detection of NCP strains of BVDV. Tanaka et al., (1975) used the test in a microtitre technique for the neutralization of NCP strains of BVDV.

Immunological methods: The immunological methods include the Ouchterlony gel diffusion technique (AGD) and the fluorescent antibody technique (FAT). Although they are most useful in the detection of NCP strains of viruses, they were also used for the detection of cytopathic strains and non-infectious antigenic particles in cell cultures or animal tissues.

Gel diffusion in BVD infection: Darbyshire (1960) observed lines of correlation between HC and BVD viruses in the double gel diffusion precipitation technique. He also reported that he could detect BVDV antigen by the technique (1962). When he examined various tissues from 223 clinically affected cattle, tissues from 85 animals revealed positive results with an immune serum and tissues from 14 animals revealed doubtful results. Tissues from 20 healthy and some normal control were all negative. Darbyshire (1967) also demonstrated the antigen in tissues of approximately 34 percent of affected animals. He described the antigen as indistinguishable from a soluble antigen produced in calf testis cells infected with Oregon C24V virus. It was stable at -20°C for at least 6 months but labile at 37°C after 90 minutes. It lost its potency within 15 minutes at 56°C and withstood lyophilisation and irradiation with ultraviolet light. Darbyshire (1967) concluded that the immunodiffusion technique provided a rapid and simple method although the sensitivity of it was not great.

Fluorescent antibody technique: Mengeling et al., (1963) used a serum-fluorescein conjugate against hog cholera (HC) antigen for the detection of cytopathic strains of BVDV in cell cultures. They also reported that the homologous serum conjugate was equally useful. Fernelius (1964) detected NCP strains in cell culture. Brown et al., (1968), Peter et al., (1968), Fernelius and Lambert (1969) and Meyling (1970) reported the use of the technique for the

detection of BVD antigen in infected animal tissues. Kniazeff et al., (1975) drew attention to difficulties inherent in the technique which he concluded were due to the unavailability of a useful BVD antiserum conjugate producing adequate and specific staining for detecting NCP strains of BVDV.

VIRUS ISOLATION BY OTHER METHODS

Trials to propagate BVDV in egg embryos failed (Olafson and Rickard, 1974; Baker et al., 1954; Hoag, et al., 1956; Huck, 1957; Underdahl et al., 1957).

Baker et al., (1954) succeeded in adapting the NY-1 strain to rabbits by three alternating calf-to-rabbit passages of splenic material from an infected animal. Fernelius, and his colleagues (1969a) also claimed adaptation of the NADL strain of BVDV to rabbits.

In sheep, Underdahl et al., (1957) did not produce lesions with their BVDV strain but they got neutralizing antibodies. Ward (1971) recovered the virus from the foetuses of experimentally infected pregnant sheep. French et al., (1974) isolated the virus from the blood of experimentally infected sheep up to the tenth day post-inoculation and procured the virus from the foetuses.

Fernelius et al., (1973) propagated a virulent strain of BVDV in pigs up to the age of 90 days.

ANTIBODY DETECTION

Serum Neutralization Test (SNT):-

Detection of neutralizing antibodies in paired sera

is considered a highly dependable method for the diagnosis of virus infections whereas a high titre of antibody in a single serum sample indicates merely either a prior infection or immunization. This generalization does not seem to hold for BVD infections. For example, Underdahl et al., (1957) in a preliminary survey of bovine sera from six widely separated herds showed that antibodies capable of neutralizing the virus were common in herds having no history of infection. Moreover Gutekunst and Malmquist (1964) isolated virus from buffy coats of animals with high titres of neutralizing antibody. In contrast Burki and German (1964) described protracted cases of viraemia with the absence of neutralizing antibodies.

Fernelius (1966) fractionated sera collected from animals immunized with a BVDV and found that the antibody in the 19S fraction predominated and persisted for as long as 20 weeks. Restimulation with the same antigen did not produce the predominantly 7S secondary response and the typical "early 19S", "late 7S" responses in virus infection were not present. The 19S did not detect viral antigen, when conjugated with fluorescein although it neutralized the virus in the serum neutralization test.

Malmquist (1968) believed that the failure of an animal with MD to develop neutralizing antibody was not uncommon. He showed that four animals with persistent viraemia failed to develop neutralizing antibodies over a period of four months and there were no precipitating and no complement fixing anti-

bodies. His explanation was that the condition probably resulted from immunologic tolerance that developed from prenatal infection. Kniazeff et al., (1967) detected neutralizing antibody in calves deprived of colostrum and Gratzek (1968) reported appreciable antibody titres in calves taken at birth by a caesarian section and deprived of colostrum. Gratzek therefore believed that there was no immunologic tolerance. This agrees with the finding of Classic and Fernelius (1970) who artificially infected a cow in the third trimester and obtained an alive calf by a caesarian section 33 days later. The calf had a high titre of neutralizing antibody although it was deprived of colostrum.

Braun et al., (1973) reported that foetuses were capable of producing antibodies by the 200th day of gestation and the presence of serum neutralizing antibodies was associated with a failure to recover the virus from foetal tissue. Nonetheless they isolated the virus from the placenta and spleen of one foetus with serum neutralizing antibody titre of 1/6500.

By far the most common form of infection was the subclinical type and it probably accounted for the high percentage of animals with serum neutralizing antibody titre (Malmquist, 1968). The duration of prenatal immunity was found to be more than 7 months in some cases and this prenatal immunity might have been responsible for the higher incidence of BVD reported in calves six to 14 months of age (Kendrick and Franti, 1974).

Snowdon (1973) reported that SNT on paired sera rarely provided information on which a presumptive diagnosis could be made. Instead examination of a single serum sample from an animal showing clinical evidence of acute or chronic BVD might provide circumstantial evidence that the disease was or was not BVD. When BVD-neutralizing antibodies were not detected in the sera of clinically infected animals, there was a high probability that BVDV could be recovered from the animal (Borgen, 1963; Bürki and Germann, 1964; Kahrs et al., 1970; Liess et al., 1974). On the other hand, if serum neutralizing antibodies were detected and particularly if the antibody titre was greater than 1/5, it was unlikely that the animal was affected with BVD (Snowdon, 1973).

Acutely ill cattle frequently died before a convalescent phase serum sample could be taken and a diagnostically significant rise in serum titres was obtained only in 21 out of 126 outbreaks of the disease (Harkness and Lamont, 1975).

The SNT was also found to be useful in the detection of naturally occurring antibodies in sheep (Bögel, 1964; French and Snowdon, 1964; St. George, 1971) and pigs (Stewart et al., 1971). Later, experimental work on sheep showed that the so called Border disease agent stimulated the production of BVD antibodies (Acland et al., 1972; Hamilton and Timony, 1973; Plant et al., 1973 and Huck et al., 1975). During the course of my studies new information on BD continued to be published (Gard et al., 1976; Plant et al., 1976; Harkness et al., 1977). According to these studies some workers

suggested that BD should be considered as a form of BVD (Gard et al., 1976; Plant et al., 1976).

In view of the difficulties encountered with carrying out the SNT in BEK cells propagated in bovine serum, Malmquist et al., (1965) adapted the NADL strain of BVD virus to primary pig kidney and PK-15 cell line and used the system for the test. Seibold and Dougherty (1967) used ovine testis cells and ovine serum for the serum neutralization test and reported that it was more reliable than bovine cells and bovine serum.

Haralambiev (1975) studied the serological relationship among three strains of BVDV and concluded that diagnostic serological testing with one strain of BVDV was unlikely to detect infected animals particularly if the antibody titre was low. Hafez et al.,(1976) tested a number of sera against six strains of BVDV and observed marked differences in the antibody titres when the sera were tested against the different strains. Some sera which gave positive results with some strains were negative with others. Consequently they recommended the use of more than one strain for routine testing. In addition to the use of more than one strain Haralambiev (1975) observed that the use of complement increased the neutralizing capacity of sera with low antibody titres and decreased the serological differences between the strains.

Agar Gel diffusion test (AGD):-

Darbyshire (1962) using a positive piece of bovine tissue as antigen obtained precipitin lines with 187 serum

samples out of 731 samples taken from BVD affected or recovered animals. Some 31 samples gave doubtful results. Sera from 115 apparently healthy animals gave three positive, one doubtful and all the rest were negative. Wachendorfer (1965) reported that a piece of small intestine from a known infected animal was useful as antigen and Darbyshire (1967) reported similar findings.

Gutekunst and Malmquist (1963) described a method for the preparation of a suitable gel diffusion antigen. They used primary BEK cell cultures started in HBSS and calf serum in five litre culture bottles. After three days, they changed the medium to EBSS and calf serum and inoculated their monolayers after five days. The cell monolayers were washed with warm PBS and inoculated with 5 ml of Eagle's basal medium containing $100TCID_{50}$ of BVDV. Adsorption was allowed to proceed at room temperature for one hour, after which maintenance medium was added and bottles were incubated at $37^{o}C$ until CPE was observed. Fluid was collected after freezing and thawing, clarified at 6500g and the soluble antigen seperated by centrifugation at 98500g for two and a half hours. The supernatant was then concentrated by pressure dialysis at $4^{o}C$ in cellulose casing. When the volume was reduced to about 15-20 ml, the casing was sealed and further concentration was achieved by "Carbowax". Darbyshire (1967) prepared AGD antigen by inoculating calf testes (CT) cells with BVDV. He then scraped the cells on the fifth day post-inoculation, sonicated them and used them as antigen.

Acland et al., (1972); Plant et al., (1973); Huck et al., (1975) and Plant et al., (1976) used BVD antigen for the detection of BD antibody in sheep and reported that it was successful.

Complement fixation (CF) test:

Although this test is one of the most valuable serological procedures available to the virologist, it has not received much attention in the diagnosis of BVD. It has never been used for the detection of BVD antigen and rarely been used for the detection of BVD antibodies. One of the difficulties was the availability of a good antigen. Gutekunst and Malmquist (1964) prepared the CF antigen by concentrating the harvest from BEK infected cultures by pressure dialysis. Ruckerbauer et al., (1971) used the same method to concentrate their antigen to one fiftieth of its original volume. They excluded phenol red from the maintenance medium as it interfered with reading the results. Eugster and Anglo (1974) concentrated their antigen by filtering the supernatant through dehydrated gel coated membrane filter impermeable to the virus particles and resuspended the particles and used them as complement fixing antigen. They also used unconcentrated antigen but they did not comment on its efficiency. Solorzano (1975) apparently used both methods of Ruckerbauer et al., (1971) and Eugster and Anglo (1974) but they did not mention whether one of the methods was better than the other. Huck et al., (1975) used the supernatant from bovine testes cell monolayers infected with the Oregon C24V strain of BVDV.

Gutekunst and Malmquist (1964) reported that in BVD complement fixing antibodies appeared earlier than serum neutralizing antibodies and persisted at a high titre for long periods. On the other hand, Ruckerbauer et al., (1971) observed that complement fixing antibodies appeared slightly later than the serum neutralizing antibodies, persisted for a shorter time and dropped to a very low level or were negative within three months. Darbyshire and Huck (1966) reported that the application of the CFT to sera from experimentally infected calves yielded equivocal results.

A titre of 1/40 was considered positive by Ruckerbauer et al., (1971). Eugster and Anglo (1974) considered a serum with 1/4 titre as negative, 1/8 to 1/16 as suspicious and 1/32 or higher as positive.

Huck et al., (1975) applied the test for the detection of border disease antibodies in naturally and experimentally infected sheep. When they compared the CFT with the SNT and AGD test, the greatest disagreement was on the group of field sera in which complement fixation failed to demonstrate antibody while the other tests did so. No explanation was given for this finding which was probably due to the fact that these field sheep had protective antibodies and were not recently infected. It is not unreasonable to suggest that CFT picks out infected rather than immune animals because complement fixing antibodies appear early and disappear more quickly than the neutralizing antibodies.

Eugster and Anglo (1974) reported that the main difficulties associated with the CFT in BVD were: firstly, some bovine sera were anticomplementary; secondly, some antigen lots were anticomplementary due to the concentration process or sera used in maintenance media; thirdly, it was difficult to concentrate the antigen without losing much of its activity and forthly, it was of no use in the diagnosis of BVD abortions as the sera might be negative at the time of abortion.

RELATIONSHIP OF THE BVDV TO OTHER VIRUSES

RINDERPEST VIRUS: (RPV)

Acute BVD and rinderpest (RP) are clinically and pathologically similar, both characterized by fever, salivation, lacrymation, diarrhoea, dehydration and extensive ulcerations of the digestive tract (Pritchard, 1963; Scott, 1964). The main difference between the two diseases are epidemiological, in particular RP in the past was associated with malignant epidemics in virgin areas. With the extensive vaccination against RP, the disease has acquired a sporadic nature and naturally less virulent strains have been observed (Wilde and Scott, 1961).

The similarities between the two diseases warranted the investigation into the immunological relationship between the two infective agents. Walker and Olafson (1947), Darbyshire (1960) and Delay and Kniazeff (1966) tried different techniques to disclose any serological relationship between the two viruses. They agreed that the two viruses were serologically distinct and neither of them bore a

serological relationship to the other.

HOG CHOLERA VIRUS: (HCV)

Trapp and Ramsey (1959) reported that cattle previously inoculated with a HC virus did not develop experimental mucosal disease and Beckenhauer and his colleagues (1961) protected HC susceptible pigs by inoculating them with Oregon C24V strain of BVDV. They also protected susceptible pigs against HC virulent challenge by injecting them with a BVD hyperimmune serum prepared in pigs. Baker et al., (1969) observed that BVDV protected pigs against HC and that colostral immunity did not interfere with immunization.

Using the Ouchterlony gel diffusion technique, Darbyshire (1960) observed an immunological relationship between the two viruses. Gutekunst and Malmquist (1963) showed that the relationship was related to a soluble antigen of BVDV which was different from the infective particle. Mengeling et al., (1963) demonstrated the relationship between the two viruses by immunofluorescence and Malmquist et al., (1965) reported that HC virus interfered with the propagation of BVDV in cell cultures. Matthaeus and Van Aert (1971) used immunoelectrophoresis to detect the relative immunological relationship. They observed that five precipitin lines were formed by HC antigen-antibody reaction. Three of those lines cross reacted with a preparation containing BVDV soluble antigen, whereas the remaining two lines were distinct for HCV.

Diderholm et al., (1973) reported the inhibition of HC virus by acriflavin, a property which was shared with BVDV. When colostrum deprived, specific pathogen free pigs were inoculated with a BVDV strain, they developed neutralizing antibodies to BVDV and HCV (Stewart et al., 1971).

BORDER DISEASE VIRUS: (BDV)

BD was first described by Hughes and Kershaw (1959) in England as a disease of lambs characterized by an increase in the amount of hair in the fleece, long and curly wool, small and fine bones, high mortality rate and central nervous system disturbances. The condition was largely confined to the progeny of young ewes although a small number of older ewes gave birth to affected lambs. The detailed pathogenesis and pathology of the disease in sheep was described by (Barlow and Dickinson, (1965); Cancilla and Barlow, (1968), Barlow and Gardiner, (1969), Barlow et al., (1970), Lewis et al., (1970), Barlow, (1972, 1972a), Carter et al., (1972), in cattle by Gibbon et al., (1974) and in goats by Gardiner and Barlow (1972), Barlow et al., (1975).

The condition was also described as "Hairy shaker disease" (Manktelow et al., 1969), "Congenital tremor", (Barlow, 1970), and "Hypomyelinogenesis congenita" (Markson et al., 1959). It was shown to be experimentally transmissible (Dickinson and Barlow, 1967; Shaw et al., 1967; and Barlow and Gardiner, 1969).

Animals which could be infected, other than sheep, were goats (Gardiner and Barlow, 1972; Huck, 1973; Barlow et al., 1975), cows (Gibbons et al., 1974) and pigs (Derbyshire, 1975).

As in BVD, infection during pregnancy, crosses the placenta and causes abortion or foetal dysmorphogenesis (Barlow and Dickinson, 1965; Barlow and Gardiner, 1969; Plant et al., 1976).

Gammaglobulins were detected in sera of precolostral lambs whose mothers were experimentally infected with BDV. (Gardiner, 1967; Patterson and Sweasey, 1969; and Barlow et al., 1970). These gammaglobulins were shown to be of foetal origin.

Although serum neutralizing antibodies to BVDV were detected in sheep (Bögel, 1964, French and Snowdon, 1964 St, George 1971), BVDV was only isolated once from a weak lamb by Hore et al., (1973). However Acland et al., (1972) inoculated sheep with BD agent and found that their immune sera reacted with BVDV in the Ouchterlony gel diffusion technique. In addition, BD antiserum was found to contain serum neutralizing antibodies to BVDV (Hamilton and Timony, 1973, Plant et al., 1973). Furthermore, Huck et al., (1975) demonstrated complement fixing and fluorescent antibodies to BVD antigen in BD antiserum. Hadjisavvas et al., (1975) demonstrated interference between BD and the NADL strain of BVDV and showed that BVD and BD immune sera neutralized the interference. Durham et al., (1975) reported that BD virus was sensitive to ether, a property

shared with BVDV. It also shared many other properties with BVDV (Vantsis et al., 1976).

BVD, BD and HC VIRUSES:

Osburn et al., (1973) detected serum neutralizing antibodies to HC virus in 13 samples taken from lambs with BD. They also detected serum neutralizing antibodies for BVDV in eight out of the 13 samples. Plant et al., (1973) reported that gel diffusion and serum neutralization tests showed similar results when they tested sera of BD cases against a BVDV. They got identical results in the gel when they used an antigen derived from an HC infected pig pancreas instead of BVD antigen. The latter workers concluded, "it would seem most likely that the three diseases are caused by viruses closely enough related to produce in their hosts antibodies which react with the soluble antigen giving the precipitin test and which neutralize an MD virus in tissue culture".

Harkness et al., (1977) detected BDV in calf testes and BEK cells by its interference with a CP strain of BVDV and the interference was neutralized by a HC antiserum. They also detected BDV with a HC antiserum fluorescein conjugate.

CHAPTER 2 Page

CRYOPRESERVATION AND CULTIVATION OF PRIMARY CELL CULTURES FOR THE PROPAGATION AND DETECTION OF BOVINE VIRUS DIARHOEA VIRUS

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INTRODUCTION

Although BVDV has been propagated in various types of cells, the favoured system is still bovine embryonic kidney cultures - (Malmquist, 1968). A major hazard, however is the presence of BVDV in embryonic cells (Classic and Fernelius, 1970), following intrauterine infection of the foetus (Gillespie et al., 1967). As a precaution against the presence of latent adventitions BVDV strains, Fernelius and Packer, (1969) recommended that BEK cells be tested by interference and immunofluorescence tests before use. An additional problem is the presence of latent viruses and interfering antibodies in commercial sera used in cell culture (Kniazeff, 1968). These complications are reflected in the different media used by workers when propagating BEK cell cultures (Lee and Gillespie, 1957; Gillespie et al., 1967).

A technique to alleviate these hazards is herein reported. It is based on the cryopreservation of BEK, lamb testes (LT) and calf testes (CT) cells while aliquots were tested for adventitious viruses.

Known uninfected cryopreserved cells served as the means by which commercial sera were tested for contamination with adventitious viruses and interfering antibodies. The technique was developed through a series of factorial experiments designed to select the optimal conditions.

MATERIALS AND METHODS

Cell Cultures:

Embryonic kidneys were obtained from foetuses of freshly slaughtered cows. After the removal of excess fat and connective tissue the kidneys were transported to the laboratory on ice in HBSS containing five percent inactivated calf sera and 1000 µg of streptomycin, 1000iu of penicillin and 25 µg of amphotericin B per ml. Testes were removed from veal calves at the time of slaughter and similarly transported to the laboratory. Lamb testes were removed surgically from neonates under complete anaesthesia.

In the laboratory, the renal capsules and medullary tissues were removed and discarded and the cortices minced. The Tunica vaginalis was removed, Tunica albuginea incised and the seminiferous tissue scraped out and minced. The minced tissues were then washed with several changes of PBS at pH 7.2. several times, until the fluid was clear. The last wash was done with prewarmed 0.25 percent w/v solution of trypsin at 37°C for 10 minutes on a magnetic stirrer. Thereafter a series of 15 minute digestion cycles with fresh 0.25 percent trypsin solution were run on a magnetic stirrer at 37°C. The supernatant was harvested at the end of each cycle and pooled in a 500 ml. flask containing about 10 ml. of inactivated calf serum to reduce any harmful effect resulting from the trypsin activity. When all the parenchymal tissue had been digested the collected tissue suspension was filtered through three layers of sterile gauze

and centrifuged at 200 g for 10 minutes. The supernatant was discarded and cells suspended in EBSS containing 0.5 percent LAH, 0.1 percent YE and 20 percent inactivated NBCS.

An aliquot of the cell suspension was tested for viability by the trypan blue exclusion test (Hanks and Wallace, 1958) in an Improved Naubauer Chamber. The cell suspension was then adjusted to give 6×10^6 cells/ml and DMSO added to a final concentration of 7.5 percent. The suspension was then dispensed in 5 ml amounts in screw-capped vials which were cooled at $-20^{\circ}C$ for 30-60 minutes before being transferred to $-114^{\circ}C$ in the vapour phase of a liquid-nitrogen refrigerator.

When stored cells were propagated, the frozen suspensions were thawed in a water bath preset at $37^{\circ}C$ and quickly diluted with 15 ml of growth medium to reduce the concentration of DMSO. For examining the growth and monolayer formation efficiencies of fresh and stored cells, suspensions were prepared in supplemented MEME (Rinaldo et al., 1976) at a concentration of 3.5×10^5 cells per ml. Roux flasks were then seeded with 80 ml, 100 ml prescription bottles with 8 ml and Leighton tubes (125 x 16 mm) containing coverslips (35 x 95 mm) with 1.5 ml. Viability counts were done on the contents of three vials and their means calculated.

For the propagation of CT and LT cells, MEMH supplemented with 10 per cent TPB (Oxoid) was used.

Cultures were maintained in MEME without vitamins and

amino-acid supplements but with 5 percent TPB. Serum supplements and other maintenance media were only used where indicated.

Serum Supplements:

LS, FCS and NBCS were obtained from the Gibco Bio-cult (Glasgow, Scotland). Sheep serum was prepared locally and sterilized by filtration. It was tested on LT cells for cytopathic effects for up to six passages before it was used for growing cell cultures on a large scale. A 5ml sample of each serum, whether commercially or locally prepared was inactivated at 56°C for 30 minutes. The aliquots were tested for neutralizing antibody at 1/4 dilutions against 100 $TCID_{50}$ of the NADL strain of BVDV. Only negative sera were used in both growth media or maintenance media, 10 percent bovine serum for growing BEK and CT cells and 10 percent ovine serum for growing LT cells.

Growth Media:

The following seven media were tried for growing BEK cells. They were supplemented with 10 percent calf serum, 100 µg of streptomycin and 100 iu of penicillin per ml.

1) EBSS supplemented with 0.5 percent LAH. (LEBSS)
2) HBSS supplemented with 0.5 percent LAH. (LHBSS)
3) Morgan, Morton and Parker 199 medium.
4) EBSS supplemented with 0.5 percent LAH and 0.1 percent YE (EYL)
5) HBSS supplemented with 0.5 percent LAH and 0.1 percent YE (HYL)
6) MEM of Eagle in EBSS (MEME)
7) MEM of Eagle in HBSS (MEMH)

Commercial suppliers of media and their constituents are given in table 1.

TABLE 1

COMMERCIAL SUPPLIERS OF MEDIA AND CONSTITUENTS OF MEDIA COMPUTED LOCALLY

Media and constituents	Commercial Suppliers
EBSS powder	Oxoid Ltd., London
HBSS powder	Oxoid Ltd., London
199 (x10)	Wellcome Laboratories, Beckenham
LAH	V.A. Howe & Co.Ltd., London
YE (Yeastolate)	Difco Laboratories, Detroit
MEME (x10) without glutamine and sodium carbonate	Gibco Bio-cult Ltd., Paisley
MEMH (x10) without glutamine and sodium carbonate	Gibco Bio-cult Ltd., Paisley
Glutamine 200 mM (x100)	Gibco Bio-cult Ltd., Paisley
MEM vitamins (x100)	Gibco Bio-cult Ltd., Paisley
MEM NEA (x100)	Gibco Bio-cult Ltd., Paisley
TPB	Oxoid Ltd., London
LHBSS	Flow Laboratories Ltd., Irvine and Gibco Bio-cult Ltd., Paisley
LEBSS	Flow Laboratories Ltd., Irvine and Gibco Bio-cult Ltd., Paisley

For the preparation of EYL, 10 g of LAH and 2 g of YE were dissolved by gentle heating in separate flasks each containing 500 ml of DDW. They were then mixed in a two litre flask and 200 ml of EBSS (x10) containing 18 ml of 1 percent phenol red were added to the mixture. The volume was then brought to 2 litres with DDW and the medium sterilized by filtration.

A 4.4 percent solution of sodium bicarbonate was prepared separately in DDW, and gassed with carbondioxide with a drop of phenol red as indicator. It was then sterilized by autoclaving at 121^{o}C for 15 minutes and 30 ml were added to the medium. This amount was found to adjust the pH of the medium at 7.1 to 7.3.

HYL was similarly prepared but with HBSS in place of EBSS and 1.4 percent sodium bicarbonate in place of 4.4 percent sodium bicarbonate.

MEM was supplemented with 1 ml of glutamine, 1 ml of MEM vitamins, 1 ml of MEM NEA and 10 ml of TPB. 2 ml of 4.4. percent sodium bicarbonate were added to 100 ml of MEME (1X) and 2 ml of 1.4 percent to 100 ml of MEMH (1X).

Evaluation of Growth Media:

Cells were propagated at three different concentrations, 1 x 10^5, 3 x 10^5 and 1 x 10^6 cells per ml and 100 ml bottles were seeded with 10 ml, 50 ml bottles with 5 ml and Leighton tubes with 1.5 ml. Cultures were then observed daily. The efficiency of monolayer formation with the different media was judged by the number and gross appearance of attached cells, the time required for monolayer formation and the extent of

monolayer cover. The attachment of cells was judged visually under the low power of an inverted microscope and the number of cells attached determined by an electronic counter (Coulter Counter, Model FN, Coulter Electronics Ltd., England) 18-20 hours after the culturing of cells. Visually observed attachment was recorded as "100" for the best media. The other mediawere recorded as 75, 50 and 25 percent as a comparative rating to the best medium. Monolayer scores were recorded five days after seeding as "4" for full monolayer cover, "3" for 75 percent cover, "2" for 50 percent cover and "1" for 25 percent cover. Cultures on coverslips were fixed in methanol, stained with Giemsa and observed under the light microscope.

For cell counts, cultures in 50 ml prescription bottles were removed with STV that contained 0.05 percent trypsin and 0.025 percent versene in normal saline. Growth medium was decanted and attached cells rinsed three times with PBS, covered with STV (both prewarmed at 37°C) and incubated at 37°C. With occasional shaking, cells were detached within 5 minutes. STV was then removed by centrifugation at 200 g for 10 minutes and the cells from each bottle resuspended in 2.5 ml of HBSS and counted electronically.

Titration of BVDV in Fresh and Stored Cells:-

The NADL strain of BVDV was passaged three times in monolayers formed from a known susceptible kidney which gave a virus titre of $10^{5.7}$-$10^{6.3}$ $TCID_{50}$/0.1 ml. The final passage was then titrated in monolayers formed from the organ to be tested. Monolayers in test tubes were rinsed with PBS

and inoculated with ten-fold dilutions of the virus up to 10^{-8} prepared in maintenance media. Three tubes were inoculated for each dilution with 0.1 ml and adsorption of the virus was allowed to proceed for 90 minutes at 37°C. Control tubes contained 0.1 ml of maintenance medium. After adsorption, 1 ml of maintenance medium was dispensed into each tube and cultures were incubated at 37°C for 7 days and the titres calculated according to the method of Reed and Muench (1938).

Evaluation of Cells Viability:-

The trypan blue exclusion test (Hanks and Wallace, 1958) was used for the determination of the percentage viability. As cells left for longer periods in the trypan blue tended to show higher percentages of stained cells, the samples were mixed with trypan blue at the time of counting. The counting was done in an improved Naubuer Chamber, 1000 cells being counted for each sample. The counting was done on three different vials and the average calculated.

In addition, three Roux flasks, one from each aliquot and nine 100 ml bottles, three for each aliquot were seeded and observed for monolayer formation. The number of days required by monolayers to form in each bottle was recorded and means and standard deviations calculated. Cultures were also propagated on coverslips in Leighton tubes, fixed, stained and observed under the light microscope for any abnormal features.

Virus Strain:

The NADL strain of BVDV of unknown passage level was

supplied by Dr. D.R. Snodgrass of the Moredun Institute. The strain was passaged five times in susceptible BBK cell monolayers and then stored at - 114°C. The stored virus had a titre of 10^6 $TCID_{50}$/ml.

Maintenance Media:

The following four maintenance media were compared; EYL, LEBSS, Medium 199 and MEME supplemented with 5 percent TPB. The four media were tried with and without serum supplements. Sera used were foetal calf serum (FCS), sheep serum (SS) and rabbit serum (RS) at the rate of 2 percent. All media contained 100 µg of streptomycin, 100 iu of penicillin and 5 µg of amphotericin per ml.

Cell suspensions in EYL growth medium were propagated in test tubes. When monolayers were formed they were washed three times with PBS and inoculated with 0.1 ml containing 100 $TCID_{50}$ BVDV diluted with the maintenance medium to be examined. Control tubes were inoculated with 0.1 ml of the maintenance medium. Tubes were then incubated at 37°C for 90 minutes to allow for adsorption and 1 ml of the maintenance medium added. Cultures were observed daily under the low power of an inverted microscope for seven days. CPE in infected cultures and degenerative changes in control tubes were recorded. Complete detachment of monolayer was scored "4", 75 percent detachment "3", 50 percent detachment "2" and 25 percent "1". Cultures with no CPE or degenerative changes were scored zero and averages calculated.

Testing of Cultures:-

Interference test: The third passage of the cultures was propagated in test-tubes which were seeded with 3.5×10^5 cells in 1 ml of MEM growth medium. Monolayers were washed with prewarmed PBS and each tube inoculated with 100 $TCID_{50}$ in 0.1 ml of MEM maintenance medium with 2 percent RS. Control tubes included uninoculated tubes to which 0.1 ml of maintenance medium was added, and inoculated tubes from known susceptible cultures. After 90 minutes of adsorption at 37°C, cultures were covered with 1 ml of maintenance medium containing 2 percent rabbit serum and observed daily until there was complete detachment of the susceptible monolayers; this normally took four to seven days.

Indirect Immunofluorescence (IFT) : The immune serum used in the IFT was supplied by Dr. D.R. Snodgrass. It was prepared by a single injection of BVDV into a gnotobiotic calf.

Cells were propagated on coverslips in Leighton tubes. Coverslips were removed, rinsed three times in PBS, dried and fixed for 10 minutes in cooled acetone. When they were dry, 1/40 dilution of the immune serum was applied to the coverslips which were placed in a humidified chamber and left at room temperature for 30 minutes. They were then rinsed for 15 minutes in three changes of PBS covered with the fluorescein conjugated rabbit-antisheep serum as recommended by Dr. D.R. Snodgrass and left in the humidified chamber for a further 30 minutes. The coverslips were again rinsed for 10 minutes in two changes of PBS and mounted with non-fluorescent immersion oil on microscope slides and examined for specific

fluorescence. In the early stages of this work, the test was performed on primary, secondary and tertiary passages of the cell cultures but later, only the third passages were examined.

RESULTS

Rate of Infection With Adventitious BVDV:

Out of the 25 bovine embryonic kidneys examined, six (24 percent) were positive by the IFT and eight (32 percent) were positive by the IT. One kidney (4 percent) showed CPE indicative of BVDV infection at the third passage level and was positive in the IFT. In all, 40 percent of the kidneys were positive.

Virus titres in uninfected kidneys varied between $\log_{10}$ 4.5 and $\log_{10}$ 7.2 $TCID_{50}$/ml (Table 2). It was observed that infected kidneys also supported the propagation of the NADL strain (Table 2). The mean titre and standard deviation of BVDV propagated in the ten infected kidney cultures was 3.59 ± 0.66 $TCID_{50}$/ per ml which was significantly lower than the mean titre and standard deviation of BVDV propagated in the uninfected cultures namely 5.47 ± 0.80 $TCID_{50}$ per ml. ($F_{25} = 5.930$. $P < 0.001$).

TABLE 2

BVDV TITRES* IN CELL CULTURES

FROM 25 BOVINE EMBRYONIC KIDNEYS

IFT and IT negative	IFT and IT positive	IT positive	IFT positive
7.2	4.8	4.1	4.5
6.5	3.8	3.8	CPE
6.2	3.5	3.5	
6.0	2.6		
6.0	2.5		
5.7			
5.5			
5.5			
5.3			
4.8			
4.7			
4.7			
4.5			
4.5			
4.5			

* expressed as $\log_{10}$ $TCID_{50}$/ml

Viability of Cells:

The mean time for the development of complete monolayers by fresh and stored BEK and CT cells was not significantly affected by long term preservation (Table 3). The time required for the formation of monolayers by LT cells progressively increased. After nine months of storage primary LT cultures failed to form complete monolayer covers but on subsequent passages they formed monolayers.

Long term preservation in DMSO did not result in adverse effects on the viabilities of BEK cells (Table 4). Significant drops were observed in CT cells examined after six months and 18 months of storage (Table 5). In contrast, the results obtained with LT cells were not consistent (Table 6). The drop in viability of aliquots tested after six months of storage was statistically significant although the drop in viability of aliquots tested three months later i.e. after 9 months storage was not. All later viability counts were significantly lower than the viabilities of fresh cells.

TABLE 3

MEAN DAYS REQUIRED FOR FORMATION OF FULL

MONOLAYER COVERS OF FRESH AND STORED CELLS

Type of Cells	Fresh	Months in storage				
		3	6	9	12	18
BEK	5.5 ± 1.4	4.8 ± 1.4	5.0 ± 1.3	5.1 ± 1.5	5.5 ± 1.3	5.2 ± 1.5
CT	5.5 ± 1.4	5.2 ± 1.1	5.1 ± 1.2	5.2 ± 1.5	4.8 ± 1.2	nd.
LT	5.3 ± 0.52	6.2 ± 1.0	8.2 ± 1.3	im.	im.	nd.

im. = incomplete monolayer

nd. = not done

TABLE 4

VIABILITY OF STORED AND FRESH BEK CELLS AS PERCENTAGE OF THE TOTAL CELLS IN SUSPENSION

Kidney	Fresh cells	Months in storage				
		3	6	9	12	18
A	52	31	48	43	56	46
B	58	-	65	50	70	56
C	60	65	43	-	65	-
D	38	36	-	35	30	37
F	76	71	68	66	-	-
G	34	40	34	-	36	-
H	33	24	37	38	-	-
Mean difference		3.83	2.67	5.00	3.00	3.00
Standard error		4.00	3.56	2.77	3.22	1.73
Student t		0.958	0.75	1.805	0.932	1.734
Probability		$<0.4>0.3$	$<0.5>0.4$	$<0.2>0.1$	$<0.5>0.4$	$<0.3>0.2$
Assessment		NS	NS	NS	NS	NS

NS = Not Significant

TABLE 5

VIABILITY OF FRESH AND STORED CT CELLS AS A PERCENTAGE OF THE WHOLE CELL SUSPENSION

CT	Fresh cells	Months in storage				
		3	6	9	12	18
A	65	66	-	55	60	54
B	88	69	79	75	65	72
C	61	58	55	67	56	53
D	63	56	53	60	60	50
Mean difference		4.51	8.00	5.0	9.00	12.00
Standard error		2.22	1.53	4.22	4.69	1.68
Student t		2.032	5.229	1.185	1.919	7.143
Probability		< 0.20 > 0.10	<0.05 > 0.02	<0.4 > 0.3	<0.2 > 0.1	<0.01 > 0.001
Assessment		NS	S	NS	NS	HS

HS = Highly Significant S = Significant NS = Not Significant

TABLE 6

PERCENTAGE VIABILITY OF LAMB TESTES CELLS AS PERCENTAGE OF THE WHOLE CELL SUSPENSION

LT Fresh cells		Months in storage				
		3	6	9	12	18
A	80	64	70	-	65	55
B	90	86	79	85	74	68
C	72	52	60	63	61	50
D	55	58	-	62	46	43
E	68	60	60	58	53	54
Mean difference		6.25	10.25	4.25	13.20	19.00
Standard error		3.55	0.85	3.90	1.36	2.53
Probability		1.761	12.059	1.090	9.706	7.510
Student t		$< 0.2 > 0.1$	$< 0.005 > 0.001$	$< 0.4 > 0.2$	< 0.001	$< 0.005 > 0.001$
Assessment		NS	HS	NS	HS	HS

HS = highly significant NS = not significant

Virus Susceptibility:

When the NADL strain of BVD was titrated in monolayers derived from stored cells, titres were not significantly different from those observed in fresh cells of BEK (Table 7), CT (Table 8) and LT cells (Table 9). Although some high titres of the virus were observed in CT and LT cells nevertheless complete detachment of monolayers at virus dilutions higher than 10^{-3} only occurred in BEK cell cultures.

Storage of cells did not affect the features of the CPE on the three types of cell cultures. CPE in BEK cells started by rounding of a few cells. This was followed by the appearance of cytoplasmic vacuoles over the whole monolayer and finally stranding and complete detachment ensued (Plate 1 and Plate 2). In rare cases intracytoplasmic inclusions (Plate 3) and intranuclear vacuoles (Plate 4) were observed. Intracytoplasmic vacuolations, stranding and monolayer detachment were also common features in affected CT cells (Plate 5 and Plate 6). In lamb testes cell rounding, stranding and detachment of cells were common (Plate 7). Pyknotic nuclei were more widespread in LT cells than in BEK and CT cells (Plate 8) but intracytoplasmic vacuolations were rare in LT cells.

TABLE 7

TITRES* OF BVDV IN FRESH AND FROZEN BEK CELLS

Kidneys	Fresh cells	Months in storage				
		3	6	9	12	15
A	6.0	5.8	-	6.3	5.7	6.6
B	6.5	6.7	-	6.0	6.4	6.7
C	3.5	3.7	4.0	-	3.3	-
D	6.0	5.8	-	6.0	5.7	6.3
E	2.5	2.2	2.3	-	-	-
F	7.2	6.8	7.0	6.7	6.5	6.5
G	5.7	5.0	5.4	5.8	6.0	5.2
Mean difference		0.20	0.050	0.12	0.18	0.14
Standard error		0.121	0.18	0.16	0.11	0.20
Student t		1.667	0.278	0.75	1.636	0.700
Probability		< 0.20 > 0.1	< 0.9 > 0.8	< 0.6 > 0.5	< 0.4 > 0.3	< 0.6 > 0.5
Assessment		NS	NS	NS	NS	NS

NS = not significant *Expressed as $\log_{10}$ $TCID_{50}/ml$

TABLE 8

TITRES* OF BVDV IN FRESH AND FROZEN CT CELLS

CT	Fresh cells	Months in storage				
		3	6	9	12	15
A	7.5	7.7	8.2	7.6	7.4	8.0
B	7.0	7.4	7.2	7.1	6.6	6.8
C	6.6	6.9	7.3	6.7	7.1	7.0
D	7.3	6.8	7.0	6.6	7.5	7.2
Mean difference		1.100	0.33	0.15	0.10	0.15
Standard error		0.204	0.24	0.05	0.19	0.176
Student t		0.490	1.375	3.000	0.526	0.852
Probability		< 0.7 > 0.6	< 0.3 > 0.2	< 0.1 > 0.05	< 0.7 > 0.6	< 0.5 > 0.4
Assessment		NS	NS	NS	NS	NS

NS = not significant *Expressed as $\log_{10}$ $TCID_{50}$/ml

TABLE 9

TITRES* OF BVDV IN FRESH AND FROZEN LT CELLS

LT	Fresh cells	Months in storage				
		3	6	9	12	15
A	6.5	6.0	6.1	-	6.8	6.3
B	6.3	6.8	-	5.9	6.2	6.7
C	6.8	6.5	7.0	6.3	7.3	6.4
D	7.1	7.4	6.6	6.3	6.5	7.3
Mean difference		0.000	0.23	0.50	0.02	0.00
Standard error		0.24	0.22	0.15	0.24	0.36
Student t		0.000	1.067	3.27	0.103	0.000
Probability		< 0.9	$< 0.4 > 0.3$	$< 0.1 > 0.05$	< 0.9	< 0.9
Assessment		NS	NS	NS	NS	NS

NS = not significant *Expressed as $\log_{10}$ $TCID_{50}/ml$

Effect of Different Media on Propagation of BHK Cells:-

Cell attachment: When cultures were observed under the low power of an inverted microscope, it was apparent that some media promoted better attachment of cells than others (Table 10) but when cell counts were done on five bottles for each medium, the differences between the media were not significant ($F_{28}^{6} = 0.33$; $P > 0.050$) (Table 11). Uniformly distributed and firmly attached fibroblastic cells were observed in MEME, EYL, HYL, LHBSS at the three levels of cells seeded. Cells in MEMH were also firmly attached but, they tended to cluster in isolated batches. In medium 199 cells were more spindle in shape and in LEBSS they were round and loosely attached to the surface. Microscopically cells in both media showed spontaneous vacuolation.

TABLE 10.

EFFICIENCY OF CELL ATTACHMENT WITH DIFFERENT MEDIA ASSESSED VISUALLY UNDER THE LIGHT MICROSCOPE

Concentration of cells seeded	Media						
	199	LHBSS	LEBSS	HYL	EYL	MEMH	MEME
1×10^6	50	75	50	100^x	100^x	75	100^x
3.8×10^5	50	75	25	75	75	50	100^x
1×10^5	nd	50	nd	75	75	50	100^x

x = The best media were rated as 100 and the others compared to it.

nd = not done.

TABLE 11

CELLS ATTACHED IN FIVE DIFFERENT BOTTLES FOR EACH MEDIUM AS DETERMINED ELECTRONICALLY

Media	Number* of cells attached					Mean + SD of cells attached	Percentage cell attached
	1	2	3	4	5		
199	1.79	1.50	0.84	1.79	1.79	1.54 $\pm$ 0.41	40.53
LHBSS	2.03	1.20	1.57	1.77	1.72	1.66 $\pm$ 0.30	43.68
LEBSS	1.69	1.73	1.08	1.59	1.61	1.54 $\pm$ 0.26	40.53
HYL	1.55	1.33	2.01	1.96	1.25	1.62 $\pm$ 0.35	42.63
EYL	2.09	1.29	2.40	1.45	1.29	1.70 $\pm$ 0.51	44.74
MEMH	2.20	1.98	1.05	2.03	1.93	1.84 $\pm$ 0.45	48.42
MEME	1.36	2.05	2.41	1.20	1.55	1.71 $\pm$ 0.50	45.00

* Number of cells are given as x 10^5 cells/ml of the original volume.

SD = Standard deviation.

Monolayer formation: Cells were cultured at three levels of concentration in Leighton tubes using 12 tubes for each dilution and the seven media under study. They were observed on the fourth day post-seeding and monolayer scores recorded (Table 12; Fig. I). The tubes were observed for ten days, growth medium being changed every three days. The days required for the formation of a full monolayer cover were then recorded (Table 13, Appendix Table A; Fig. II). LHBSS medium tended to become acidic very quickly especially with high levels of cells seeded. LEBSS became deep red when incubated and was too alkaline for growing cells. The conclusions were as follows.

(i) Differences between levels of cells seeded were not significant ($F^{2}_{207} = 2.43$; $P > 0.050$.

(ii) The interaction between cells and media were not significant ($F^{12}_{207} = 1.28$; $P > 0.050$.

(iii) There were significant differences between media ($F^{6}_{207} = 18.69$; $P < 0.010$.

Further analyses by Duncan's multiple range test yielded three significant subsets of media, two of which overlapped, the best subset being EYL and MEME (Fig. III and Appendix table B).

TABLE 12

MEAN MONOLAYER FORMATION SCORES FOR BEK CELLS SEEDED AT 3 LEVELS IN 7 MEDIA USING 12 REPLICA

Concentration of cells seeded/ml	199	LEBSS	LHBSS	EYL	HYL	MEME	MEMI
1×10^6	2.08	2.42	2.50	3.75	2.42	3.83	2.42
3.8×10^5	2.00	2.00	2.67	3.33	3.25	3.83	2.25
1×10^5	nd	nd	1.67	3.00	2.75	3.50	2.00

nd = not done

TABLE 13

DAYS REQUIRED FOR FORMATION OF MONOLAYER BY THE DIFFERENT MEDIA

Concentration of cells seeded/ml	199	LEBSS	LHBSS	EYL	HYL	MEME	MEMH
1×10^6	7-10	5-10	5-7	3-5	4-6	3-5	6-10
3.8×10^5	8-10	d	6-8	4-6	4-6	3-5	7-10
1×10^5	nd	nd	7-10	5-7	5-8	4-6	7->10

d = cells degenerated and medias continued to be too alkaline

Microscopically vacuolations were observed in cultures propagated in medium 199 and LEBSS, which also failed to form complete monolayer covers. In LHBSS and HYL media, intercellular spaces were observed. Moreover, results obtained with LHBSS were not consistent. Overcrowding of cells was observed in EYL and MEME when cultures were left in the growth media after the monolayers were formed.

Maintenance Media:

Rabbit and sheep sera were both better than FCS in the maintenance media tried when observed for seven days (Fig. IV). The uninfected cultures maintained with FCS showed far more cytopathogenic changes than the control cells maintained with sheep or rabbit sera. On the other hand, less degenerative changes were also observed in cells maintained in EYL and MEM especially when they were supplemented with sheep or rabbit sera. Regression analysis indicated that the best medium/serum combination was MEME/ sheep or rabbit serum, and the worst combination was LEBSS/FCS (Fig.V).

MEME supplemented with TPB also gave good protection to the uninfected cell cultures up to 5 days after inoculation and no medium change was necessary.

DISCUSSION

My observations indicated that the end-points of virus titrations were more easily read on BEK than on CT

or LT cell cultures. This property makes BEK cell monolayers more suited for the SNT. BEK cell cultures were also the cultures of choice for the isolation of BVDV as primary kidney cells derived from the same species were necessary for the efficient detection of infective viruses (McFerran et al., 1972). In areas with high BVD incidence, it is very difficult to obtain bovine embryonic kidneys which are not infected with BVDV. Out of the 25 kidneys examined (Table 2) ten (40 percent) showed virus by the interference test, fluorescence test or both.

Sera which are used for cell cultures, including foetal calf sera, may be contaminated with antiviral substances or interfering viruses (Kniazeff et al., 1967; Swack et al., 1975). Such sera can confuse the results by giving false positives if they are contaminated with viruses. They can give false negative results if they contain interfering antibody. They may also help to conceal very low titres of virus in contaminated cell cultures.

It was found that BEK cells could be processed and cryopreserved as primary cells without any deleterious effect on their propagation efficiency (Table 3 and 4) or susceptibility to BVDV (Table 8). Such preserved uninfected cells dramatically reduced the time required for BVD diagnosis as they saved the time required for the collection, processing and testing. They also removed

the frustration encountered with the unavailability of the bovine embryonic kidneys. In addition they were invaluable as a means by which serum supplements could be tested before being used for growing or maintaining cell cultures.

Many different media have been used for growing BEK cell cultures. This is probably a reflection of the inefficiency of the different media in promoting cell growth and maintenance when they are supplemented with different batches of serum. Of the media selected and studied, MEME supplemented with TPB gave the most consistent growth with different batches of bovine serum. An advantage of this medium was that it was chemically defined. Another advantage of the MEME was that it could be used without serum supplement for maintaining BEK, CT and LT cells. If the cells were to be maintained for more than five days, the addition of 2 percent rabbit or sheep serum was found to abolish the need for changing the medium (Fig.IV).

Fig. I MEAN MONOLAYER - FORMATION SCORES FOR BEK CELLS.

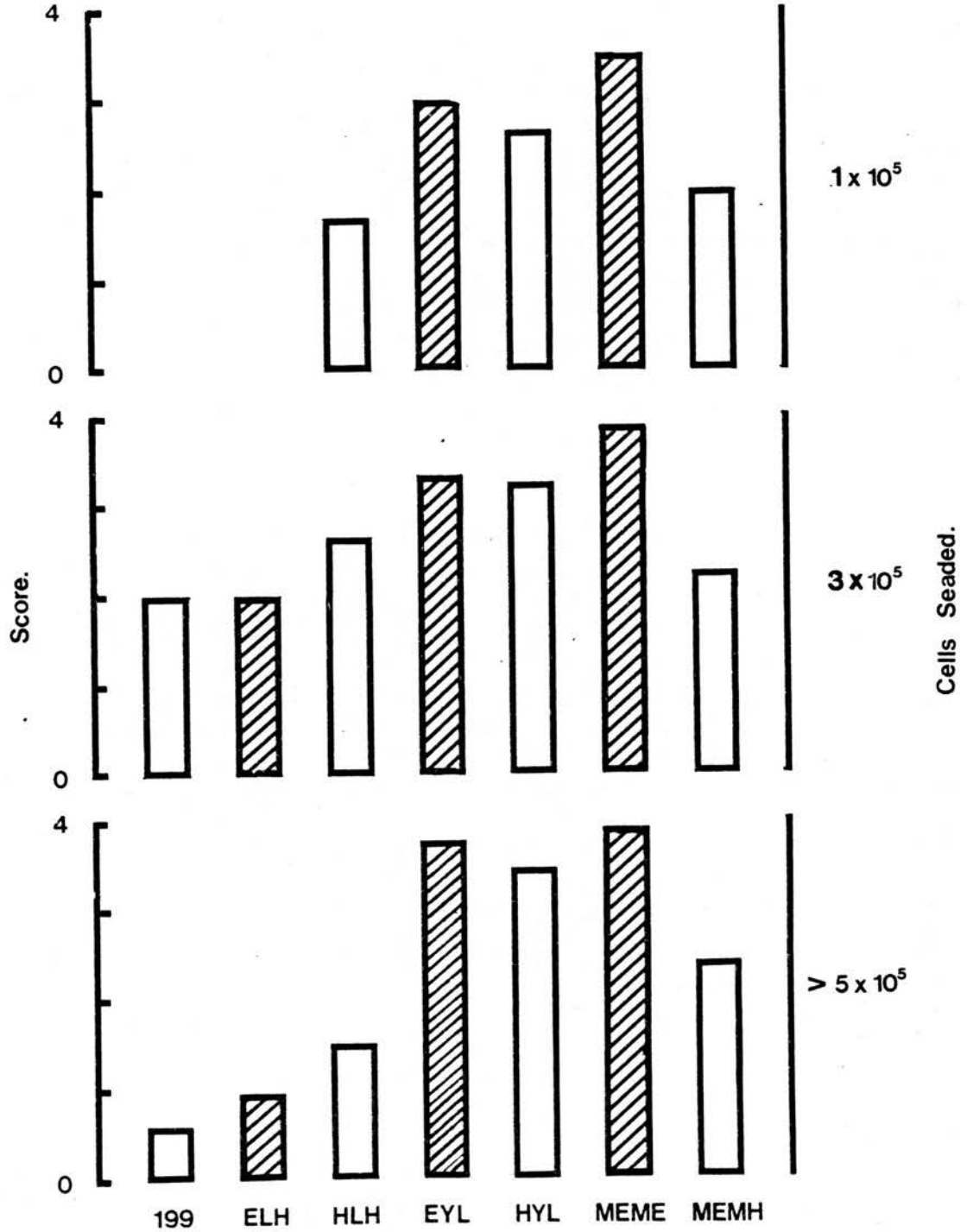


Fig II DAYS REQUIRED TO FORM MONOLAYERS.

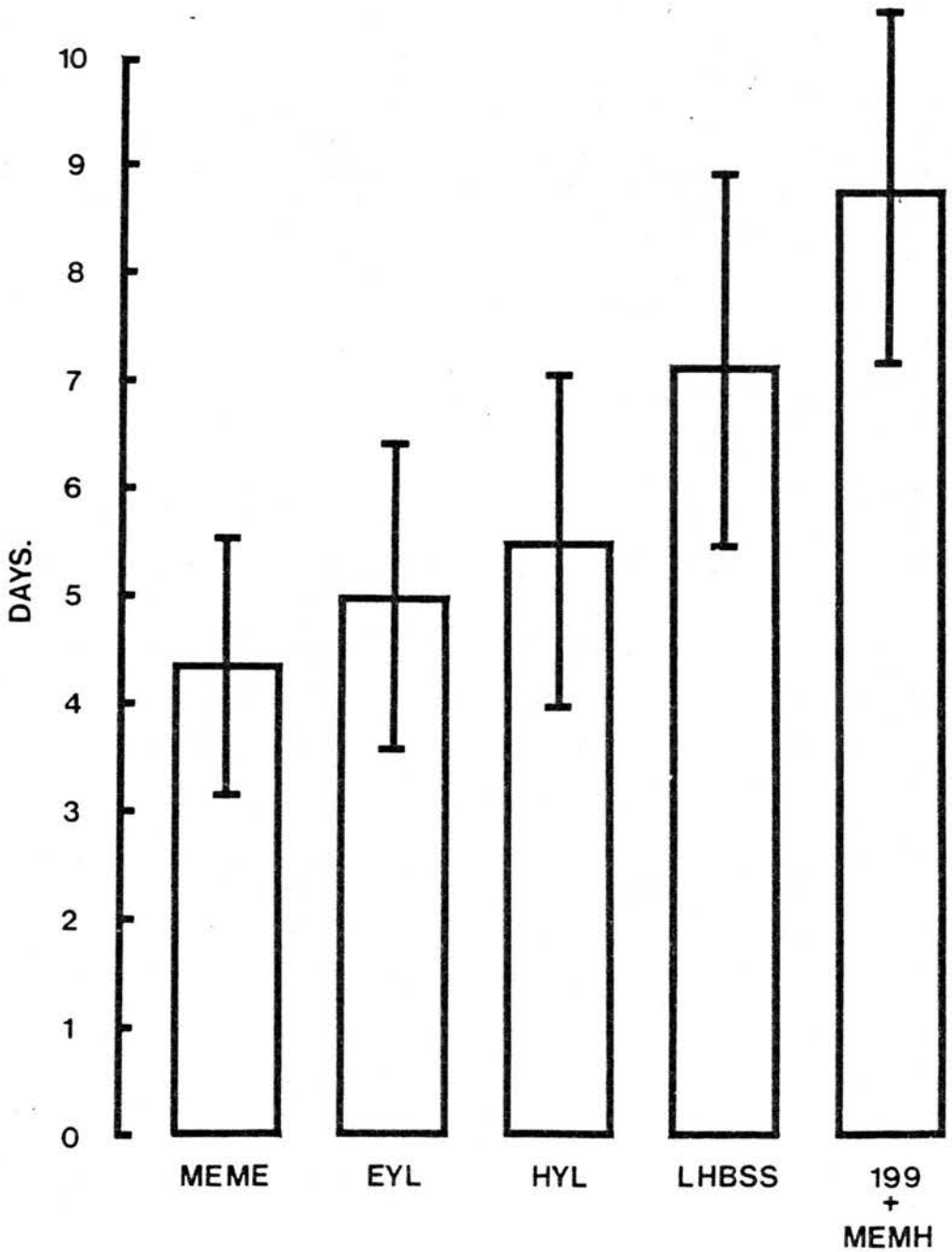


Fig III MONOLAYER-FORMATION SCORES : SIGNIFICANT SUBSETS OF MEDIA.

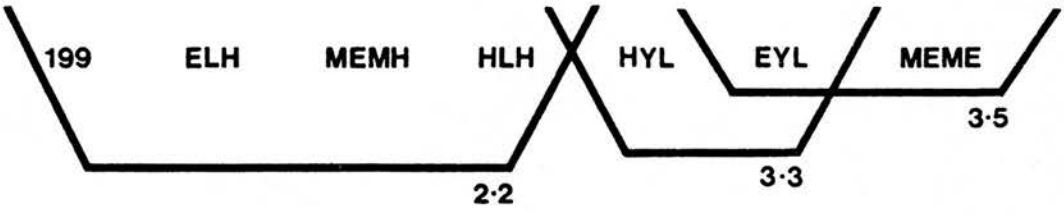
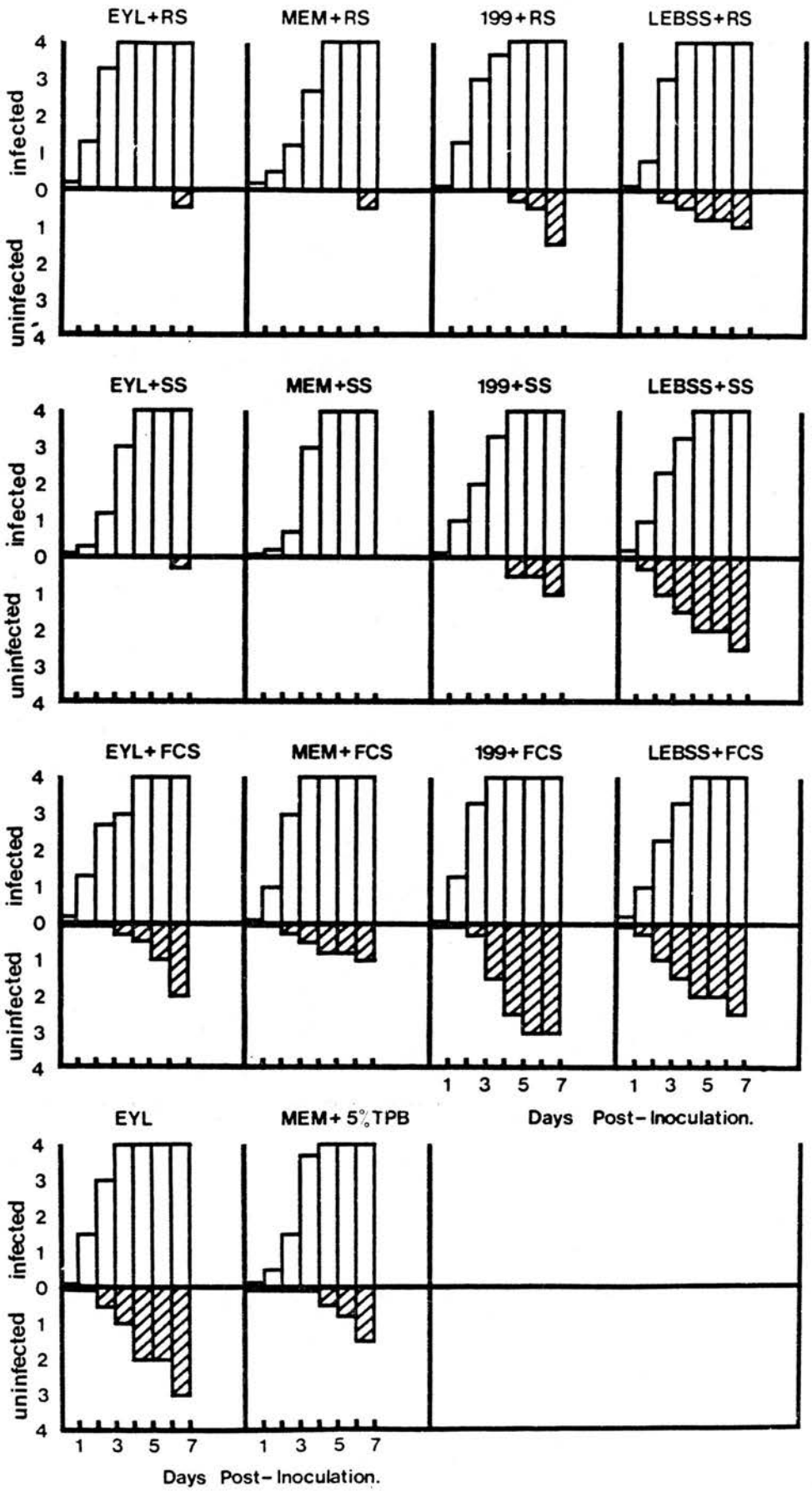
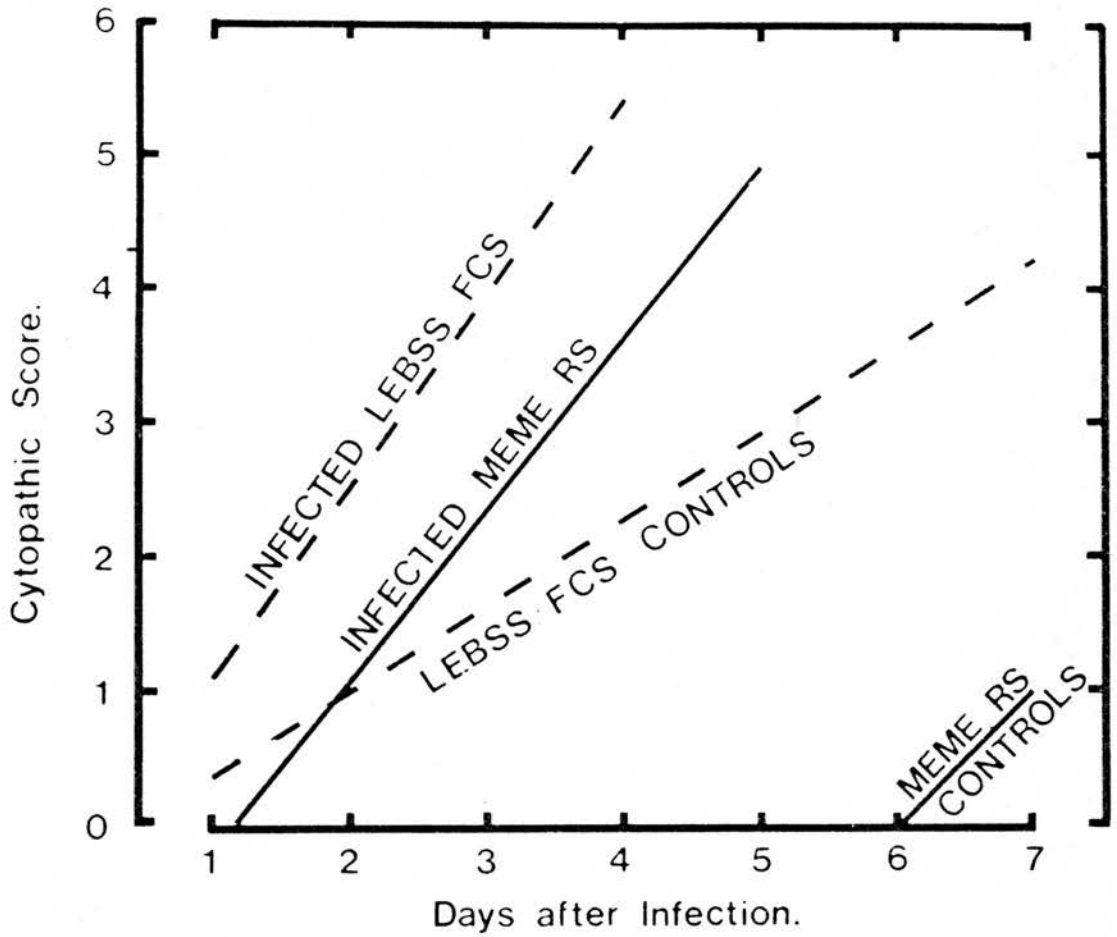


Fig. IV. Cytopathogenic changes in cells infected with the NADL strain of BVDV and in uninfected cells maintained in four different media supplemented with three different sera.

Fig IV


EYL+RS
MEM+RS
199+RS
LEBSS+RS
EYL+SS
MEM+SS
199+SS
LEBSS+SS
EYL+FCS
MEM+FCS
199+FCS
LEBSS+FCS
EYL
MEM+5%TPB
infected
uninfected
4 3 2 1 0 1 2 3 4
1 3 5 7
Days Post-Inoculation.

FigV



CPE IN BVDV-INFECTED AND NON-INFECTED BEK CULTURES: BEST AND WORST MEDIUM AND SERUM COMBINATIONS.

PLATE 1. BEK cell monolayer propagated in MEME

X500

PLATE 2. BEK cells infected with the NADL strain of BVDV showing pyknotic nuclei (a), intracytoplasmic vacuolations (b) and stranding (c)

X 500

PLATE 1.

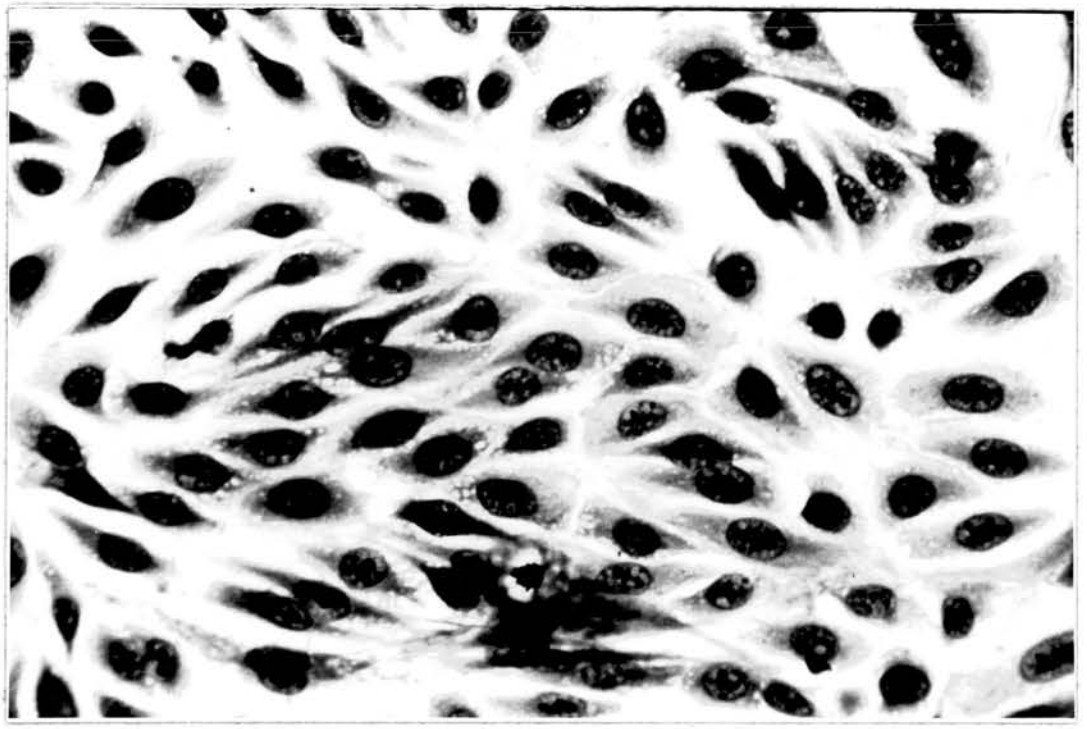


PLATE 2.

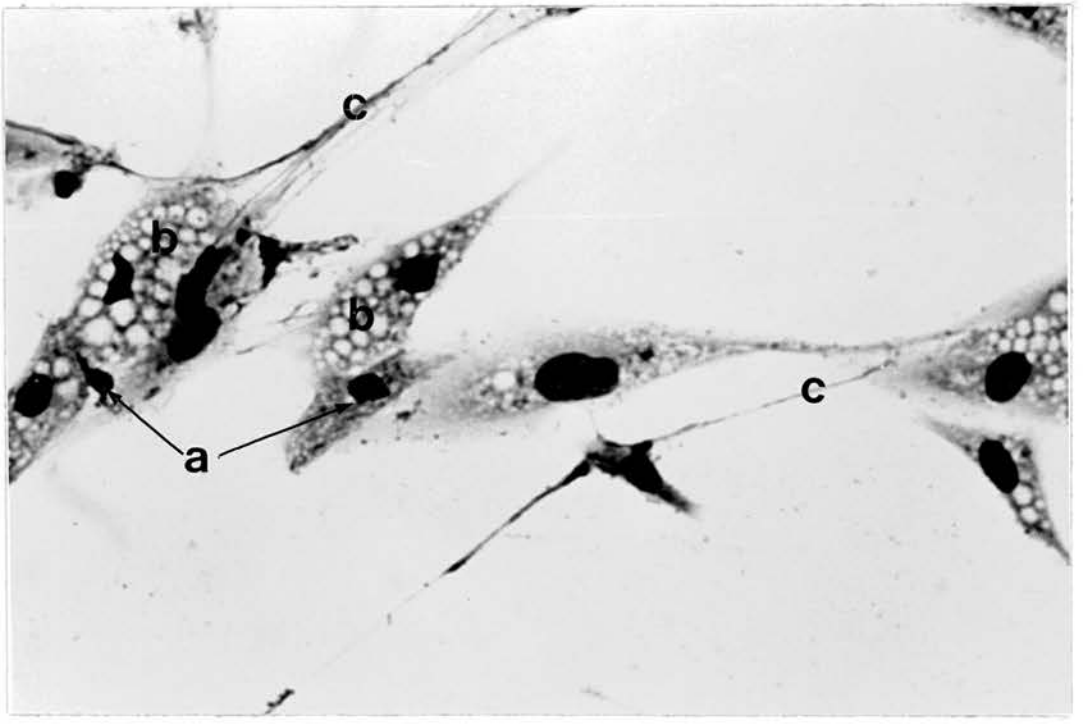


PLATE 3. BEK cells infected with the NADL strain of BVDV showing intracytoplasmic inclusions (d) X 2000

PLATE 4. BEK cells infected with the NADL strain of BVDV showing intranuclear vacuoles (e) and nuclear karyorrhexis (f) X 800

PLATE 3.

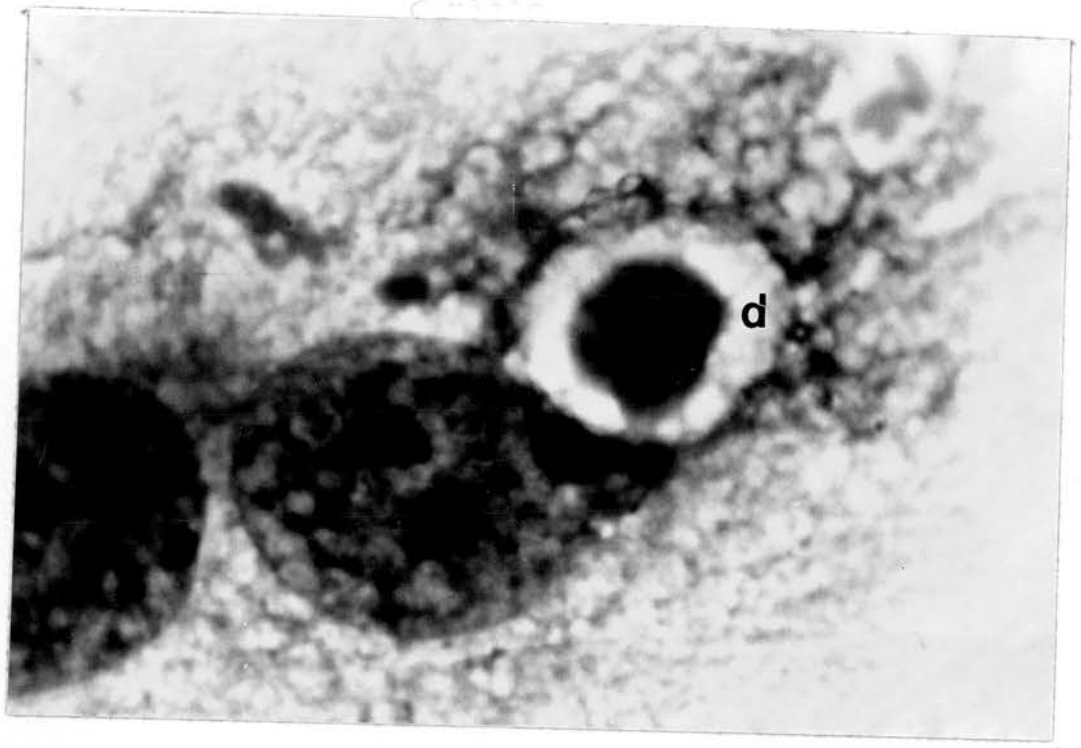
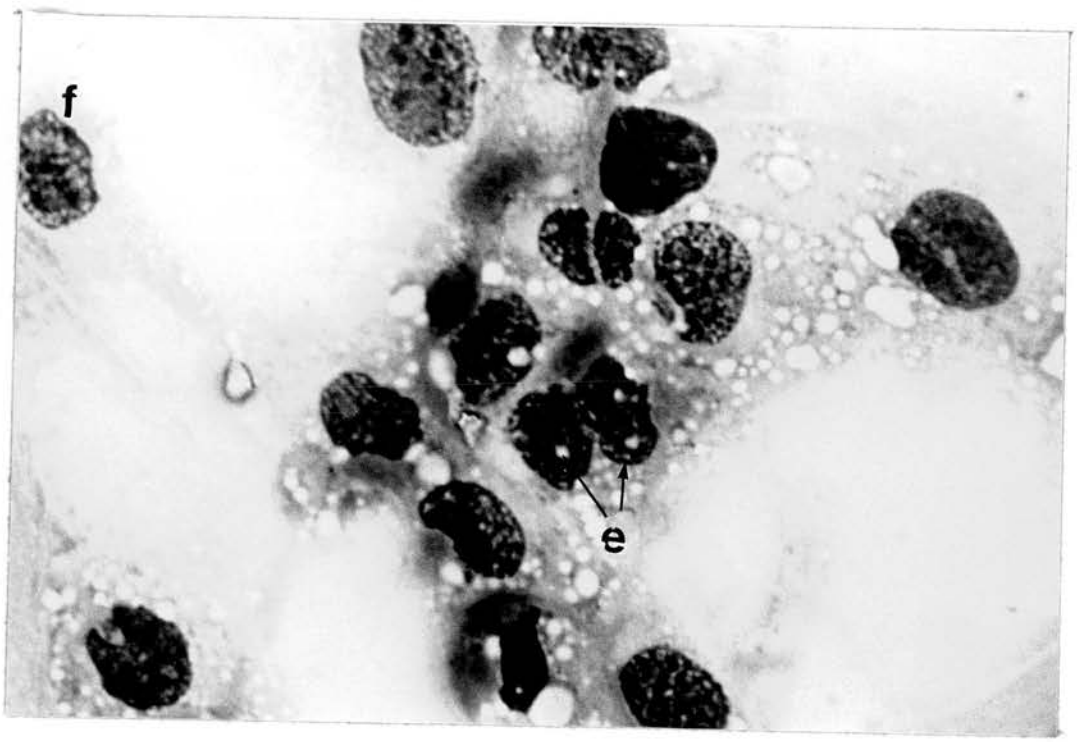


PLATE 4.



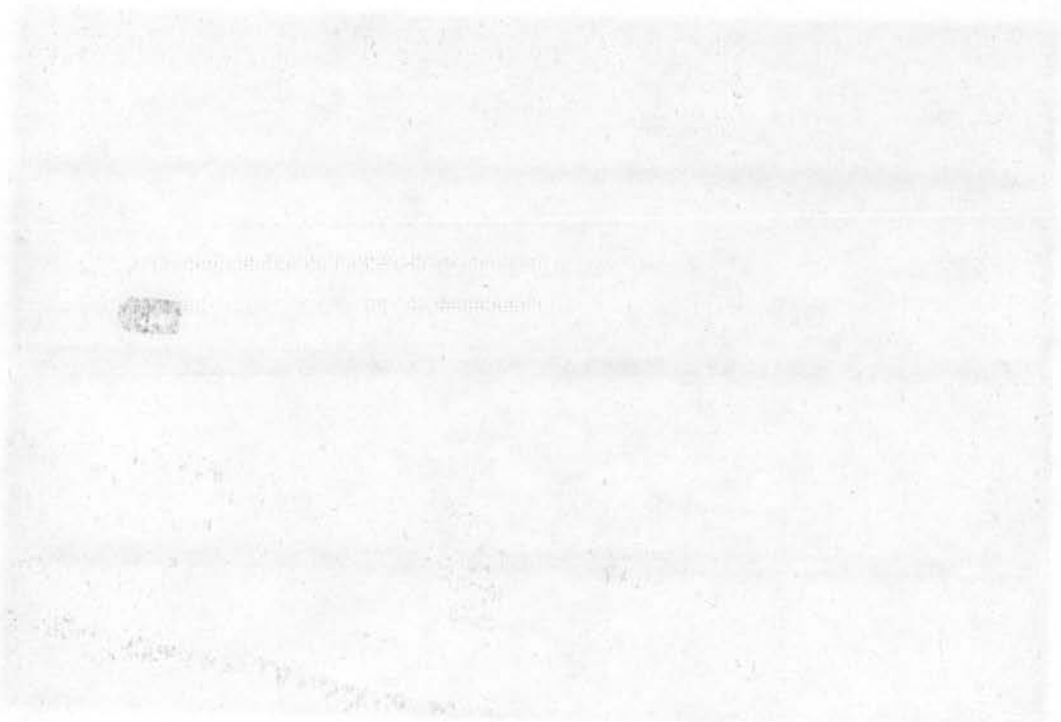


PLATE 5. CT cells in a monolayer propagated in MEMH X 500

PLATE 6. CT cells infected with the NADL strain of BVDV showing intracytoplasmic vacuolations (h), stranding of cells(i) and pyknotic nuclei(j) X 500

PLATE 5.

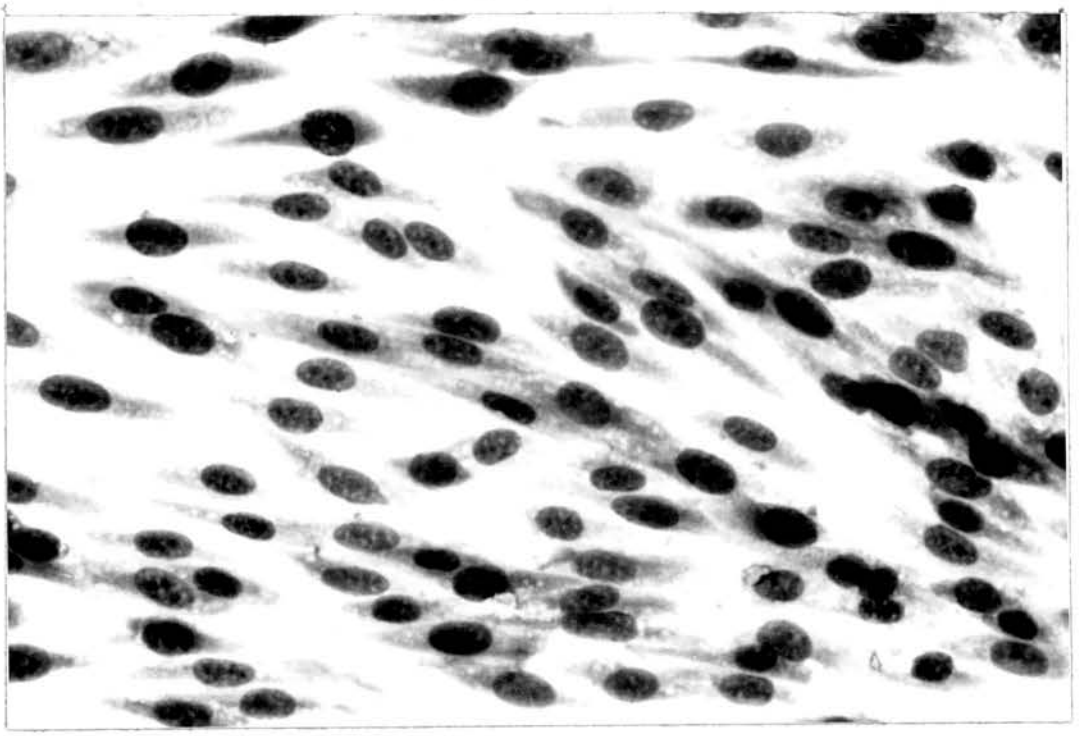


PLATE 6.

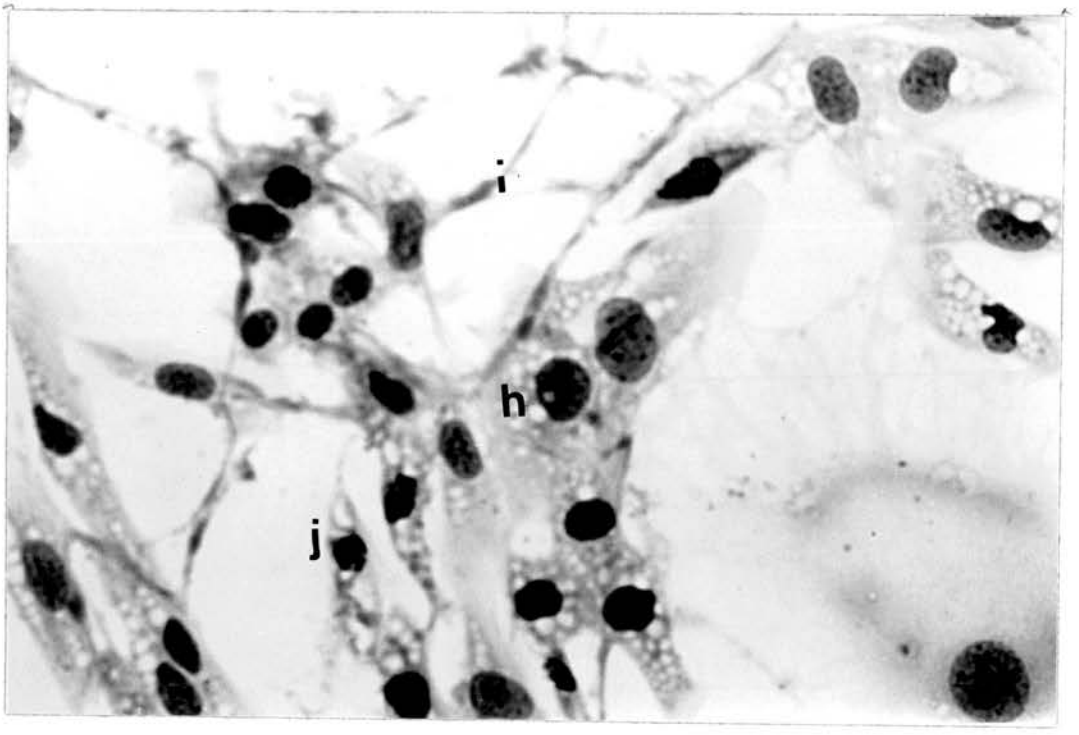


PLATE 7. LT cell monolayer propagated in MEMH

X 200

PLATE 8. LT cells infected with the NADL strain of BVDV showing pyknotic nuclei(k), stranding (m) and intracytoplasmic vacuolations(p)

X 200

PLATE 7.

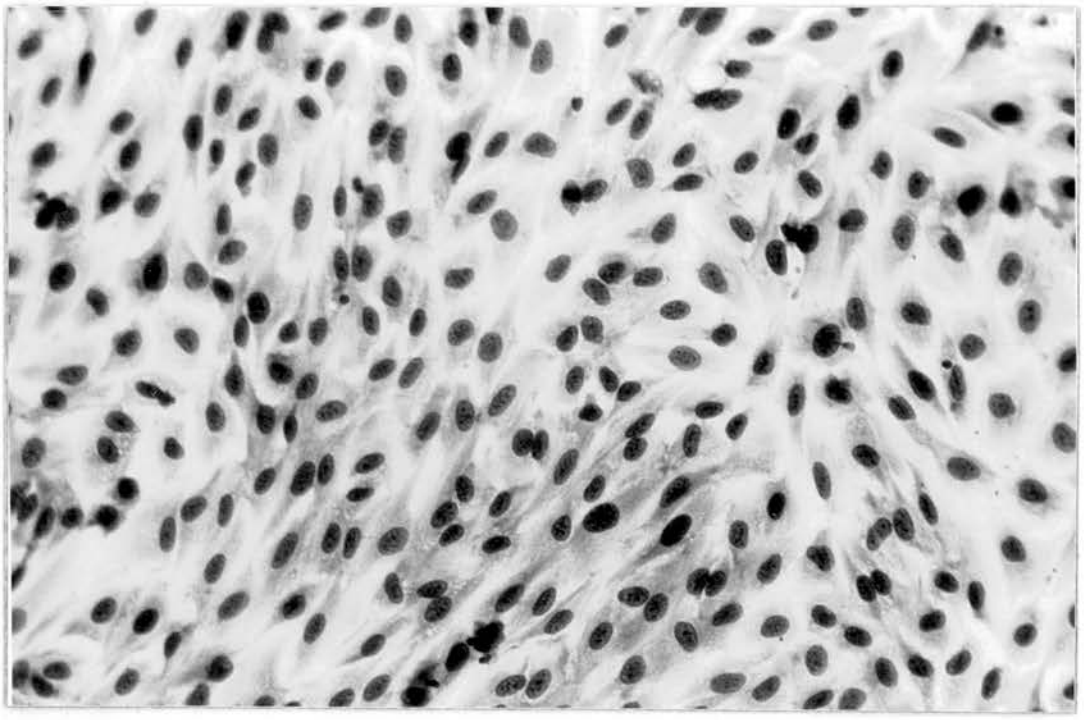
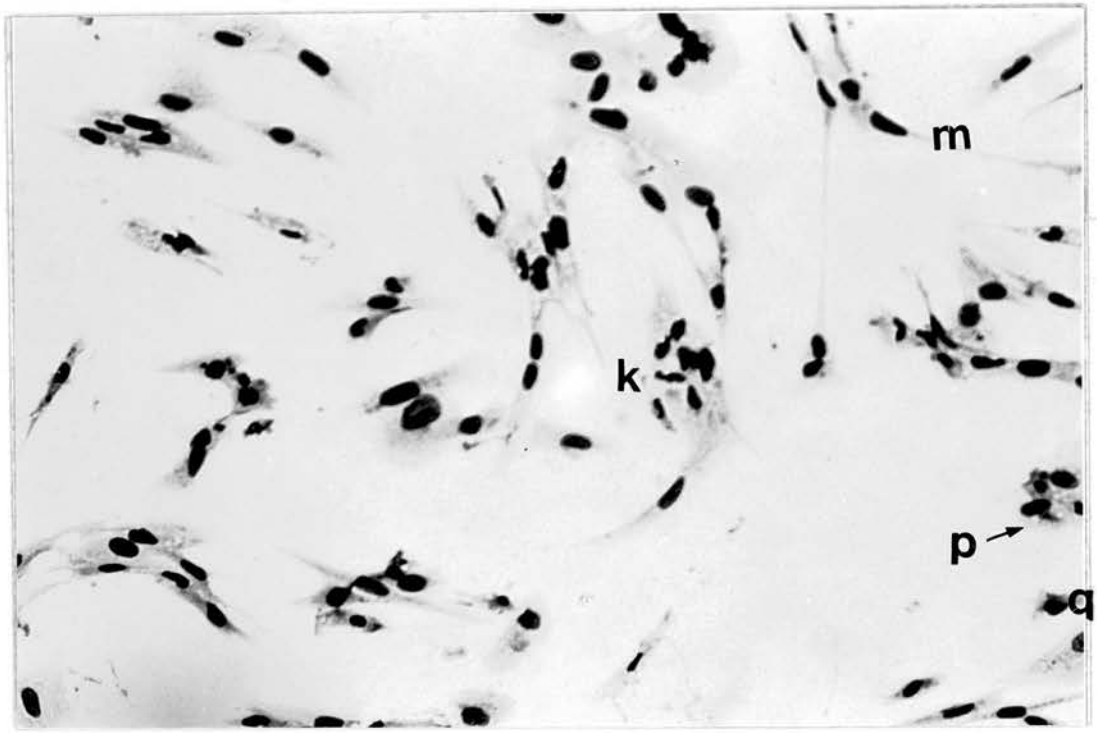


PLATE 8.



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CHAPTER 3

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INTRODUCTION

Many strains of BVDV are non-cytopathic (Gillespie, 1968). Further techniques are therefore necessary to detect the presence of BVDV in cell cultures. Both the standard interference (Gillespie et al., 1962) and the "enhanced Newcastle disease" (END) tests (Inaba et al., 1963) have proved to be unreliable (Plant et al., (1976). Most diagnostic laboratories now favour the detection of specific intracellular antigens by immunofluorescence, a technique that requires the preparation of monospecific sera conjugated with fluorescein.

As yet there is no BVDV antiserum conjugate that is satisfactory for detecting BVDV antigen in cell cultures. Antisera prepared in rabbits reacted with both uninfected cells and cells infected with BVDV (Fernelius and Packer, 1969). Kniazeff et al., (1975) tried different calf antisera used by different laboratories for the detection of BVDV and concluded that, at best, BVD was a poor antigen for fluorescent antibody studies. Another feature of the BVD-fluorescent antibody test was the marked difference in the intensity of fluorescence among the different strains (Heuschele, 1975).

MATERIALS AND METHODS

Preparation of Antigens:-

BEK and LT cells were maintained in MEME supplement with 5 percent TPB. Two percent rabbit or sheep serum

were added when the antigen was prepared for the inoculation of rabbits or sheep respectively. No serum supplement was used when the antigen was intended for the inoculation of the calf.

Inoculated and control cultures were harvested when there was 80 percent CPE or more in the infected cultures. Cell cultures were harvested by three cycles of freezing and thawing in a mixture of dry ice and methylated spirit. The harvest was centrifuged at 1500 g for 15 minutes to remove the cell debris. The virus titre of the supernatant fluid derived from infected BEK and LT cells ranged from $10^{5.0}$ to $10^{6.5}TCID_{50}$ per ml. The inoculum for sheep, was concentrated fifty times using "carbowax"[1] and partially purified by being run through a Sephadex G-200 column[2]. The fluids from the three peaks (Appendix Fig. 1) were collected separately and titrated in cell cultures. The first peak which gave the most virus was concentrated and used for inoculating sheep.

Animals:-

Rabbits: Antisera were produced in ten New Zealand White rabbits. Two rabbits were inoculated with the NADL strain of BVDV propagated in BEK cells (NADL-BEK), two with fluid from BEK cells, three with the NADL strain propagated in LT cells (BVD-LT) and three with fluid from LT cells. The immunisation schedule was as follows:
Each rabbit was bled and then inoculated with 5 ml of the fluid

1) Gurr biological Reagents, Chadwell Heath

2) Pharmacia, Uppsala

by the subcutaneous route and one ml by the intravenous route. Then 1 ml was given intravenously, weekly for four successive weeks, the rabbits being bled two weeks after the last injection.

Sheep: Four serologically negative sheep were selected for immunization. Two sheep were inoculated intranasally with partially purified BVD-LT antigen, each being infected by the intranasal instillation of 10 ml of the virus fluid which contained $10^4 TCID_{50}$ per ml after concentration. Six weeks later two similar doses were given intravenously on two successive weeks and the sheep bled two weeks after the last injection. The two other sheep were similarly inoculated, one with BEK and one with LT cell-clarified fluids.

Calf: A calf which had a very low titre of BVD antibody (1/2 per 100 $TCID_{50}$) was given an intravenous injection of $10^6 TCID_{50}$ of the NADL strain propagated in BEK cells. Six weeks later it was given two similar injections on two successive weeks and bled seven days after the last injection. On the same day it was injected with $10^4 TCID_{50}$ of a non-cytopathic strain of BVDV and bled two weeks later.

Collection of Sera:-

The calf and sheep were bled from the jugular vein and the blood samples were allowed to clot at 37°C for 1 hour. They were then centrifuged at 1500 g. The clarified sera were collected and stored at -20°C.

Rabbits were sedated and their ears thoroughly cleaned. A marginal ear vein was exposed and punctured and the whole ear inserted into a vacuum bleeder[1] which allowed the collection of 50 ml of blood without causing shock. The clarified sera were then stored at -20°C.

Virus Strains:

The cytopathic NADL strain and a noncytopathic strain of BVDV isolated from an infected BEK (NCP BVD2) were used. The NADL strain was also used in the microtitre neutralization test for the detection of BVD antibodies in immune sera. The cytotoxicity of the immune sera was also determined by the microtitre technique.

Complement Fixation Test:

The antigen used for testing the rabbit, sheep and calf sera were harvests of the NADL strain of BVDV propagated in BEK, CT and LT cell cultures. Control antigens were the harvests of the homologous uninoculated cell cultures. The medium for maintaining cell cultures did not contain serum supplement.

Sera were mixed with similar volumes of $^{1}/_{10}$ guinea-pig complement and allowed to react overnight in the cold to neutralize anticomplementary effect (Grist et al., (1974). They were then inactivated at 56°C for 30 minutes before they were tested.

Complement fixation was carried out in WHO haemagglutination plates. Overnight fixation was allowed to proceed at 4°C in the presence of 2.5 units of complement.

1) Belco Biological Glassware, Vineland

This was followed by one hour incubation at 37°C on a shaker after the addition of the haemolytic system. The highest dilutions giving complete fixation of complement were taken as the end points.

Gel Diffusion Test:

The gel diffusion plates used for testing the serum contained 0.5 percent agar in distilled water. Sera were tested before and after absorption with sheep liver powder at the rate of 0.1 gm. of tissue for each ml of serum (Nairn, 1976). Absorption was carried out at 4°C overnight. The antigens used were BVDV propagated in BEK, and LT cells and the homologous control cells.

Labelling of Sera:

Gamma-globulins in sheep and calf sera were precipitated and conjugated with fluorescein isothiocyanate (FIT)(1) according to the method described by (Nairn, 1976).

After three cycles of precipitation, the globulins were concentrated by "Carbowax" and dialysed overnight against three changes of PBS.

Titration of Labelled Antisera:

BEK cell monolayers on coverslips in Leighton tubes were inoculated with 100 $TCID_{50}$ of the NADL strain or a noncytopathic strain of BVDV. Adsorption was allowed to proceed for 90 minutes at 37°C, the maintenance medium added and tubes incubated for 24 hrs. Uninoculated tubes were used as controls.

Coverslips were then removed, rinsed in PBS, dried, and fixed in acetone for 10 minutes. They were allowed to dry and then the conjugate was applied to them. Dilutions of

(1) Gurr Ltd., London.

the conjugate used ranged from neat to $^{1}/_{40}$. Coverslips were left in a humid chamber at room temperature for 30 minutes and then washed for 15 minutes with three changes of PBS. They were rinsed in distilled water and mounted on microscope slides with U.V. immersion oil(1). Coverslips were examined under a fluorescent microscope.

(1) Vickers Ltd., York.

RESULTS

Neutralizing Activity of Sera

Pooled antisera produced in rabbits to BVDV propagated in BEK (BVD-BK) and LT (BVD-LT), as well as the control BEK and LT cells, neutralized the NADL strain of BVDV in both BEK and LT cells (Table 14). BVD-BK antiserum gave the highest titre (1/640), followed by BVD-LT and BEK antisera (1/160) and finally LT antiserum (1/20).

Absorption of the antisera with powdered ovine liver tissue did not result in an appreciable drop in the titres of the virus antisera. Although there was some drop in the titres of control sera, the BEK antiserum still neutralized $100TCID_{50}$ at 1/40 dilution. The titre of the LT cells antisera dropped to less than 1/5 and in a separate titration it was found to range between 1/2 and 1/4 ($\log_{10}$ 0.5) (Table 14). Antisera to BEK and BVD-BEK were cytotoxic at low titres, the cytotoxicity of the virus antiserum being higher (Table 14).

Pooled sheep antiserum to BVD-LT had a titre of 1/640 and the calf serum had a titre of 1/1280. Sera from sheep inoculated with LT or BEK uninfected cultures did not neutralize the virus (Table 14).

TABLE 14

THE NEUTRALIZATION AND TOXICITY TITRES OF ANTISERA PRODUCED IN DIFFERENT ANIMALS

Antisera	Antigen used for immunization			
	BVD-BEK	BEK	BVD-LT	LT
Rabbit antiserum	640(x)	160	160	20
" " tissue adsorbed	320	40	160	< 5
" " cytotoxicity	20	5	0	0
Sheep antiserum	-	0	640	0
Calf antiserum	1280	-	-	-

(x) Given as the reciprocal of the serum dilution

Complement-fixing Activity of Sera:

The rabbit sera produced to the NADL strain propagated in BEK (BVD-BK), LT (BVD-LT) as well as the BEK and LT cell control antisera fixed complement with the virus strain propagated in BEK, LT and CT cells and with the uninoculated control cells (Table 15). The highest CF titre was 1/1024 and was produced by the reaction of BVD-BK antiserum with homologous virus suspension. Generally, the titres of BVD-BK antiserum and BVD-BEK antigen tended to be higher even when tested against the other antigens and antisera respectively. The virus suspensions also seemed to fix more complement with most of the antisera than the homologous control uninfected cells except when calf testes cells were used; the virus and cell control titres were then similar (Table 15).

Although the calf serum had a high titre of neutralizing antibody it failed to fix complement. Most sheep sera likewise failed to fix complement; low antibody was only detected in sheep BVD-LT antisera with BVD-BK and BEK antigens (Table 15).

TABLE 15

COMPLEMENT FIXATION TITRES(x) OF ANTISERA PRODUCED IN DIFFERENT ANIMALS AGAINST VIRAL ANTIGENS AND THE CORRESPONDING CELL CONTROLS.

Antisera	Antigens					
	BVD-BK	BEK	BVD-LT	LT	BVD-CT	CT
Rabbit BEK	256	64	64	32	64	64
" BVD-BK	1024	128	512	128	256	128
" LT	512	16	128	32	32	32
" BVD-LT	512	32	256	32	32	64
Sheep BVD-LT	8	4	0	0	0	0
Calf BVD-BK	0	0	0	0	0	0
Sheep LT	0	0	0	0	0	0
Sheep BK	0	0	0	0	0	0

(x) Given as the reciprocal of the serum dilution.

Gel Precipitation Reactions:

Virus and cell control antisera produced in rabbits reacted with the virus antigen whether produced in BEK or LT cells (Plate 9 and 10; table 16). When the sera were absorbed with liver tissue, virus antisera continued to show the precipitation reactions but the reactions of the control sera varied; it was either absent (Plate 9) or weak to the extent that it was only observed when photographed (Plate 10).

The reactions with the control antigens were somewhat different; virus antisera showed strong double or three line reactions, but the anticellular sera reacted only with the corresponding cell antigen (Plate 11). When sera were tissue-abosrbed all the reactions disappeared except for a weak reaction between BVD-LT antisera and the corresponding cell control (Plate 11). Antisera produced in sheep and a calf did not show any precipitation reactions.

TABLE 16

GEL DIFFUSION REACTIONS BETWEEN ANTISERA PRODUCED IN RABBITS AND VIRAL AND CELLULAR ANTIGENS

Antisera	Antigens			
	BEK	BVD-BEK	LT	BVD-LT
Rabbit BEK antiserum	+	+	-	+
" " " absorbed	-	±	-	±
" BVD-BK antiserum	+	+	+	+
" " " absorbed	-	+	-	+
" LT antiserum	-	+	+	+
" " " absorbed	-	-	-	±
" BVD-LT antiserum	+	+	+	+
" " " " absorbed	±	+	±	+

± = Weak or variable reaction

Fluorescence Activity of Sera:

Sheep Serum: Trials with conjugated sheep serum on infected and uninfected cell cultures showed that it did not detect specific BVDV antigen. The background fluorescence was intense when the serum was used at dilutions up to $^{1}/10$. At higher dilutions the fluorescence was very weak and not specific.

Calf Serum: The concentrated calf globulin contained 3.5 g per 100 ml at the final stage of precipitation with ammonium sulphate. When the precipitate was conjugated it was found to show specific fluorescence with the NADL strain of BVDV in cell cultures at $^{1}/10$ dilution. The fluorescence was intense and easily detectable (Plate 12) but was less intense with a noncytopathic strain of BVDV (Plate 13). Challenging the calf with the latter strain did not improve the intensity of fluorescence. Moreover, the fluorescence was even more difficult to detect when another cytopathic strain of BVDV was inoculated into cell cultures.

The fluorescence with the NCP strain was either diffuse in the cytoplasm around the nucleus or granular (Plate 13).

DISCUSSION

Confirmation of a provisional diagnosis of BVD is seldom attempted by detecting specific antibodies because at least five weeks must elapse after the clinical episode for CF antibodies to be detectable (Gutekunst and Malmquist 1964). The use of paired sera also requires a lengthy period.

The availability of monospecific antiserum hastens the confirmation of a diagnosis by identifying specific antigen in cell cultures, the immunofluorescence test being often used (Heuschele, 1975). Sera produced in rabbits showed peculiar reactions which made them unsuitable for such a sensitive test. Antivirus sera as well as anti-cellular sera neutralized the virus. They also fixed complement with the viral antigen as well as the cellular control antigen. Virus antisera reacted with the viral antigens as well as the control cellular antigens in the gel diffusion test even after they were adsorbed with liver powder. Cellular antisera reacted with the homologous cellular antigen only, but they reacted with the viral antigen produced in the homologous as well as the heterologous cells.

Serum produced in sheep did not show non-specific reactions in the serum neutralization or the gel diffusion tests. Nevertheless it was not useful in the fluorescent technique because of the intensity of the background fluorescence.

A useful serum for the detection of BVD antigen was produced in a calf. The intensity of fluorescence varied with the different strains of the virus. Challenging the

calf with a heterologous strain did not improve the intensity of fluorescence with that strain. This was probably due to the fact that by the time of the challenge, the level of antibody had already attained its maximum titre. A possible answer to this problem is the use of a multi-valent antiserum.

The findings published by Fernelius (1966), provide another explanation for the weak fluorescence intensity. The author reported that the antibody in the 19S fraction persisted for as long as 20 weeks in BVD antisera which were prepared in calves. Even when the animals were challenged the predominantly 7S response did not occur. The 19S antibody did not detect BVDV antigen by fluorescence.

In my experiment, although the calf was bled nine and 11 weeks after the first injection, the 19S antibody was probably still predominant. This possibly led to competition between the 19S and 7S antibodies in their reaction with the antigen resulting in weak fluorescence.

PLATE 9 Immuno-diffusion reactions between antisera produced in different animals and the NADL strain of BVDV propagated in BEK cells:

(A) Rabbit BEK-antiserum, (a) Tissue adsorbed rabbit BEK-antiserum, (B) Rabbit BVD-BEK-antiserum (b) Tissue adsorbed rabbit BVD-BEK-antiserum (D) Rabbit LT-antiserum (d) Tissue adsorbed rabbit LT-antiserum (E) Rabbit BVD-LT-antiserum (e) Tissue adsorbed rabbit BVD-LT-antiserum (F) sheep BVD-LT-antiserum (G) calf BVD-BEK-antiserum (R) Virus propagated in BEK cells.

PLATE 9

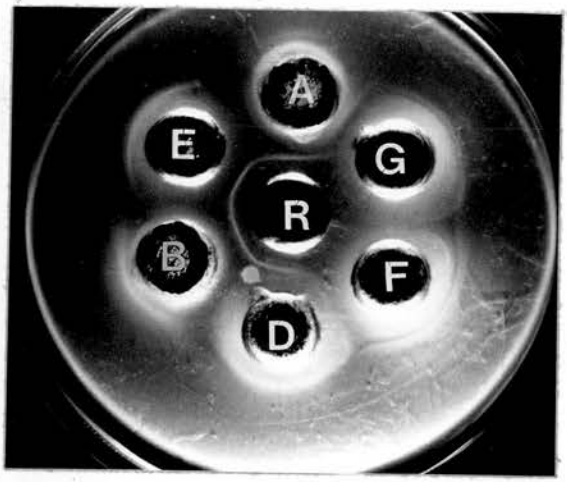


PLATE 10. Immuno-diffusion reactions between antisera produced in rabbits and the NADL strain of BVDV propagated in BEK and LT cells.

(A) BEK-antiserum (a) Tissue adsorbed BEK-antiserum (B) BVD-BEK-antiserum (b) Tissue adsorbed BVD-BEK-antiserum (D) LT-antiserum (d) Tissue adsorbed LT-antiserum (E) BVD-LT-antiserum (e) Tissue adsorbed BVD-LT-antiserum (R) BVDV propagated in BEK cells (T) BVDV propagated in LT cells

PLATE 10

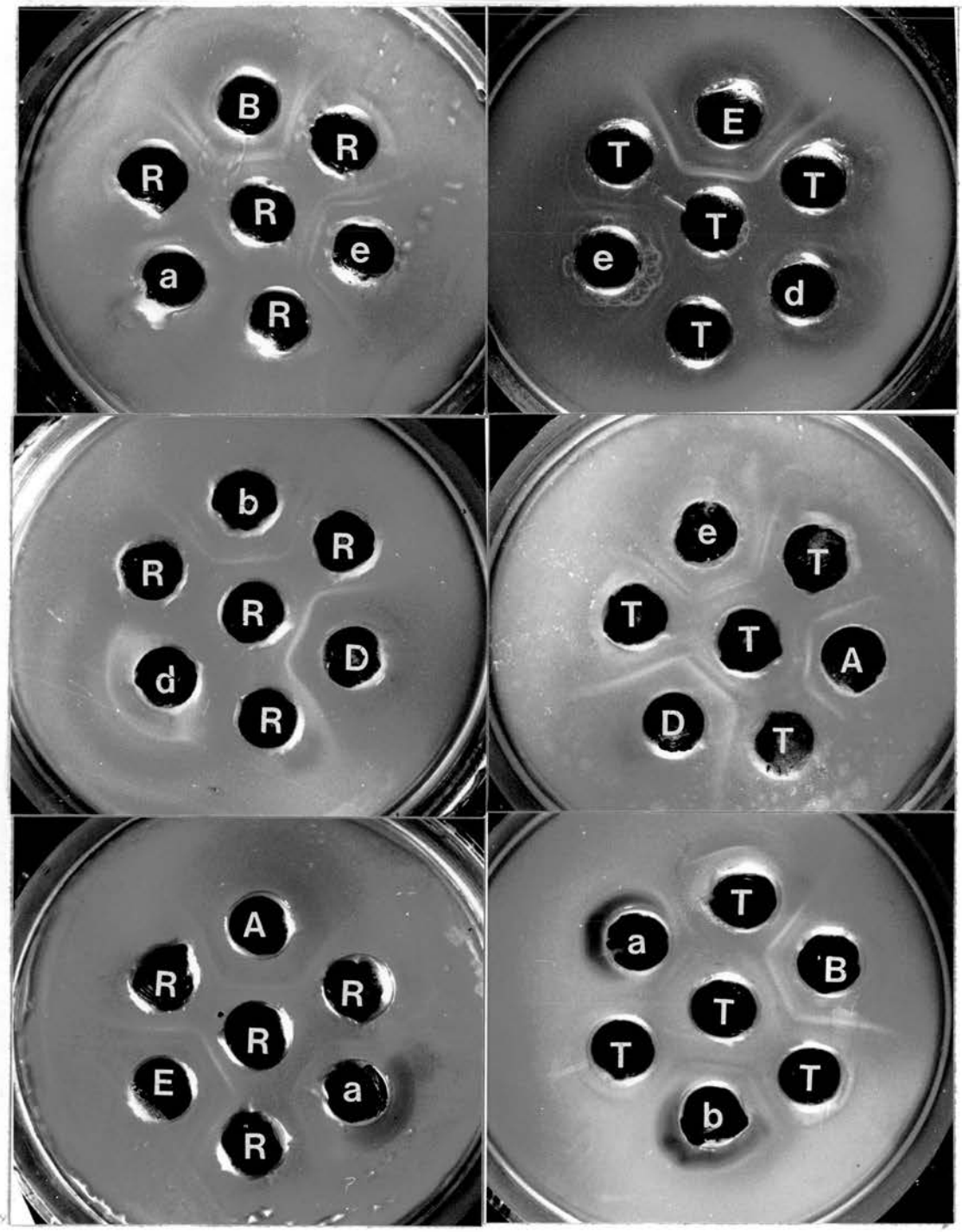
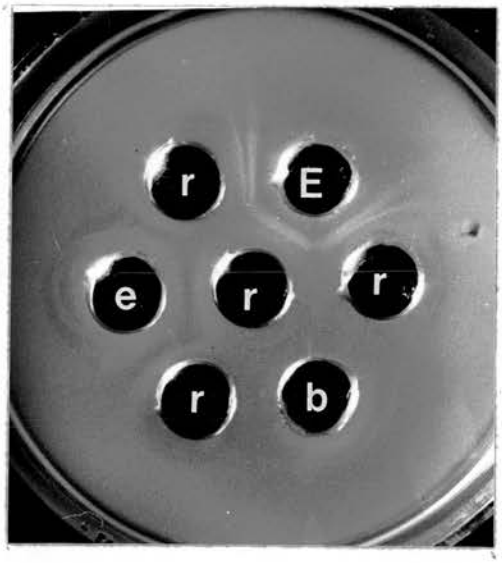


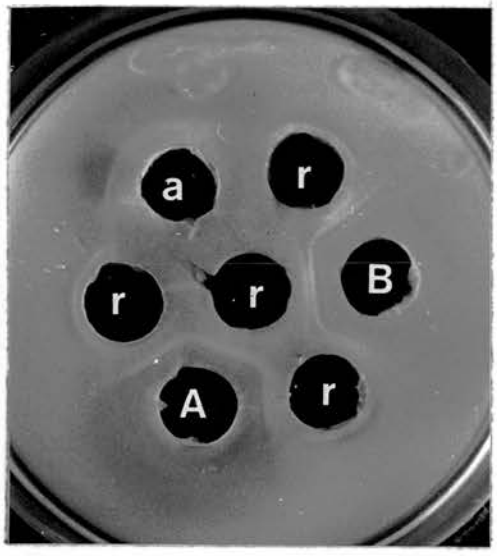
PLATE 11. Immuno-diffusion reactions between antisera produced in rabbits and uninfected BEK and LT cells

(A) BEK-antiserum (a) Tissue adsorbed BEK-antiserum (B) BVD-BEK-antiserum (b) Tissue adsorbed BVD-BEK-antiserum (D) LT-antiserum (d) Tissue adsorbed LT-antiserum (E) BVD-LT-antiserum (e) Tissue adsorbed BVD-LT-antiserum

(r) BEK cells (t) LT cells

PLATE 11





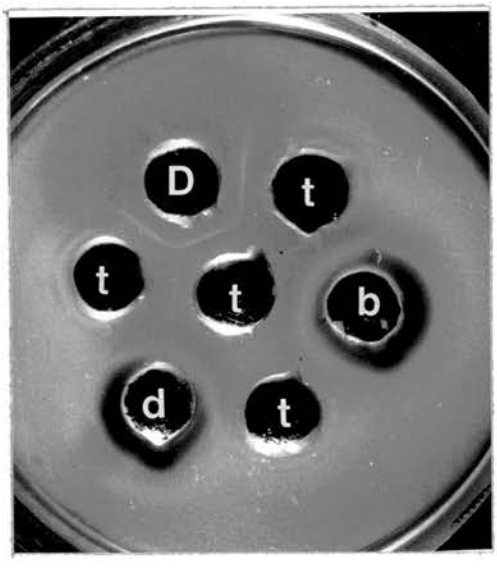
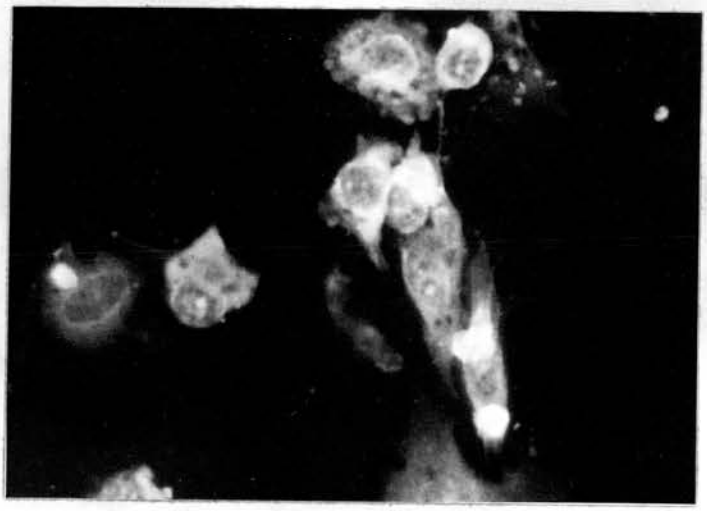
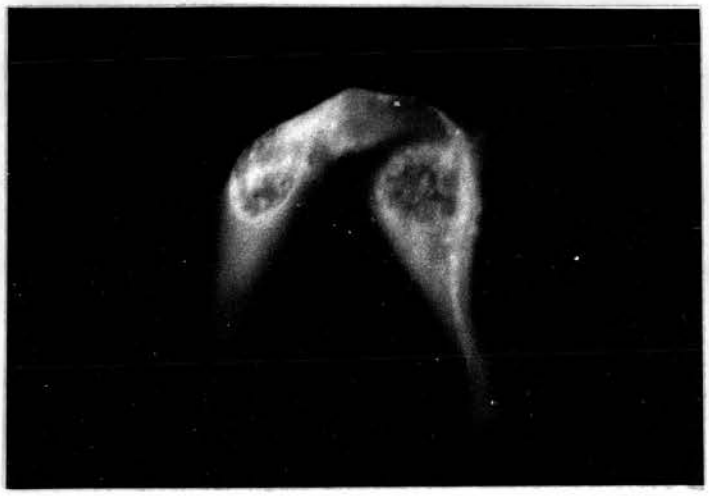


PLATE 12

FLUORESCENCE REACTION OF THE NADL STRAIN OF BVDV



X 1200



Oil immersion

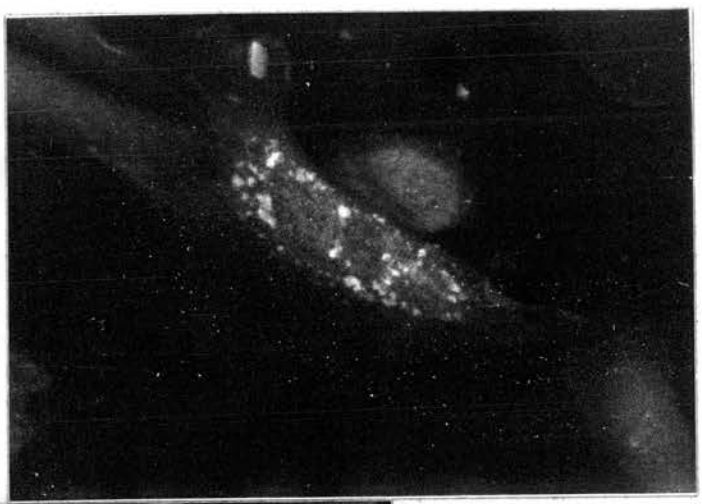
X1800

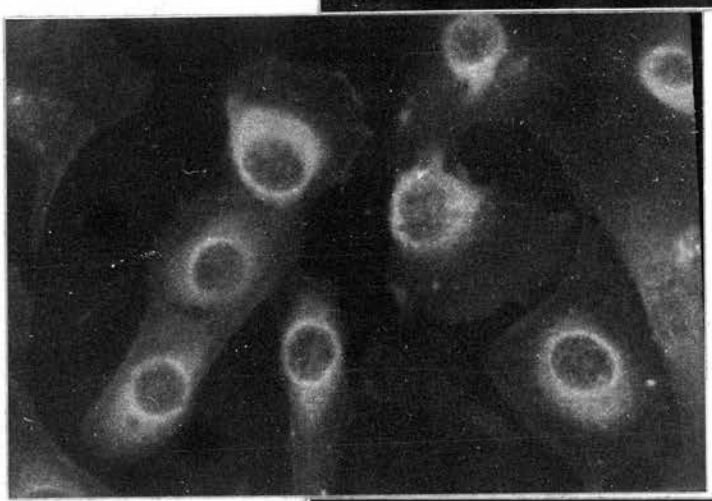
PLATE 13

FLUORESCENCE REACTION OF A NCP STRAIN OF BVDV

Granular

X 1200





Diffuse

X 1200

Oil immersion

X 1800

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INTRODUCTION

Underdahl et al., (1957) successfully produced antisera to BVDV by injecting sheep which developed neither pyrexia nor any other clinical reactions. When pregnant sheep were inoculated with BVDV, a mild febrile reaction occurred and abortions, stillbirths, foetal autolysis and mummifications were observed (Ward, 1971; French et al., 1974); Snowdon et al., (1975). Nervous disorders in newborn lambs also occurred (Ward, 1971). Recovery of the virus was achieved from whole blood, kidneys, livers, spleens, foetal membranes and pooled viscera of foetuses and lambs after experimental infection of the pregnant sheep (French et al., 1974).

Natural infection of sheep with BVDV was suspected when specific neutralizing antibodies were detected in field sera (Bögel, 1964; French and Snowdon, 1964). More recently the virus was isolated from the liver of a lamb that died immediately after birth (Hore et al., 1973). Sheep were also shown to become naturally infected when they were kept with infected cattle (French et al., (1974).

Acland et al., (1972), Hamilton and Timony (1972), Gard et al., (1976) and Plant et al., (1976) associated BVDV with border disease due to the similarities between the two conditions and their causative agents.

I inoculated non-pregnant sheep with BVDV as a model for mild infection to find out the best procedure for a quick and efficient method of diagnosis.

MATERIALS AND METHODS

Sheep:

A variety of breeds and their crosses were used including Blackface, Finn and Dorset. To select seronegative sheep, sera were tested in the SNT at $^{1}/_{2}$ dilutions.

In virus isolation and antigen detection trials, eight sheep were inoculated intranasally and a similar number were inoculated intravenously each sheep receiving a dose of $10^{6}TCID_{50}$ of the NADL strain propagated and titrated in LT cell cultures.

To assess the risk of contagion among sheep, two uninoculated animals were housed with two sheep inoculated intranasally. Similarly two uninoculated sheep were housed with two infected intravenously.

Eighteen seronegative sheep were inoculated with different doses of BVDV to determine the minimum dose inducing seroconversion. Four sheep were inoculated with $10^{4}TCID_{50}$, four with $10^{3}TCID_{50}$, four with $10^{2}TCID_{50}$, four with 10 $TCID_{50}$ and two with 1 $TCID_{50}$ of the NADL strain of BVDV propagated in LT cells.

Cell cultures:

BEK, LT and CT cells were propagated in test-tubes at the third passage level in MEME supplemented with 10 percent TPB and 10 percent antibody free calf serum for bovine cells or 10 percent lamb serum for LT cells. For the

SNT, BEK cells were used because they were found to give easily readable end-points. For the microtitre neutralization test, the third passage of BEK cells was suspended in MEME supplemented with 10 percent TPB and 5 percent sheep serum. Maintenance media for the tube cultures did not contain serum.

Sera:

Sheep were bled into 10 ml vacutainers[1]. After blood had clotted, the serum was clarified by centrifugation and stored at - 20°C. When sera were to be tested, they were inactivated at 56°C for 30 minutes.

Samples For Virus Isolation:

The earliest samples were collected on the fourth day postinoculation and every four days thereafter up to the 20th day post-inoculation. Samples collected for virus isolation were as follows:

Whole blood: blood was collected in sterile vacutainers containing heparin and 0.1 ml of blood were inoculated into each of 5 tubes of BEK cells, 5 tubes of CT cells and 5 tubes of LT cells.

Nasal and rectal swabs: four swabs were collected from each animal, one from each nostril and two from the rectum. Swabs from the nostrils were homogenized together and similarly the duplicate rectal swabs. Homogenization

1 Becton, Dickenson and Co., New Jersey.

was performed with a Griffin Vortex stirrer (S37-850) in 2 ml of maintenance medium containing 200 iu of penicillin, 200 µg of streptomycin and 10 µg of amphotericin per ml. In addition 2 µg of gentamicin sulphate per ml were added to eliminate contaminating mycoplasmas. The swabs were then removed and the fluid clarified by centrifugation at 2000g for 15 minutes in a cold centrifuge. Thereafter samples were inoculated into tubes as described for blood samples.

Virus Isolation And Identification:

Inoculated cultures were incubated at 37°C for 90 minutes to allow adsorption to take place. Tubes were then rinsed twice with PBS and 1 ml of maintenance medium added into each tube. They were observed daily and cultures which did not show CPE were passaged into fresh cultures between the 3rd and 5th days post-inoculation.

After the third passage, negative tubes were challenged with 100 $TCID_{50}$ of the NADL strain to test for interference.

Samples from four of the sheep infected intranasally and from the eight sheep inoculated intravenously were examined for BVDV specific fluorescence using BVDV anti-serum conjugate of bovine origin. At each passage cover-slips were removed, rinsed with PBS, fixed in acetone and treated with the serum. At least two coverslips were tested for samples which did not show CPE and one coverslip from samples which showed CPE. Coverslips with extensive CPE were avoided.

Virus isolates were also confirmed as BVDV by neutralization with $^{1}/_{40}$ dilution of a calf BVD antiserum.

The Microtitre Serum Neutralization Test (MTSN)

For the dilution of sera, dilution of virus and preparation of cell suspension, MEME supplemented with 10 percent TPB and 5 percent antibody free sheep serum and flat-bottom, deep cup plates[1] were used.

As a preliminary screen, all the sera were tested at $^{1}/_{2}$ dilutions and two-fold dilutions prepared from every positive serum. Each dilution was transferred to four cups using a drop volume of 0.05 ml per cup. An equal volume of the NADL strain of BVDV containing 100 $TCID_{50}$ was mixed with the serum dilutions and the mixture allowed to react at 37°C for 60 minutes. Control wells either contained two drops of medium or one drop of medium and one drop of virus.

The plates were then removed from the incubator and one drop of BEK cell suspension prepared at a concentration of 6×10^{5} cells per ml was added to each well. The plates were covered and incubated at 37°C in a humid atmosphere adjusted to have 5-10 percent carbon dioxide. Plates were observed until complete CPE developed in the virus controls, normally five days after preparation. When complete CPE had developed, the results were read and the titres of the sera calculated according to the method of Reed and Muench (1938)

1 Nunclon, Gibco Bio-cult Ltd., Scotland.

Virus Titration:

Blood samples collected from sheep which were inoculated by the intravenous route and which showed viral CPE on primary isolation, were titrated in tubes. Ten-fold dilutions were prepared and 0.1 ml of each dilution was mixed with 1 ml of cell suspension containing 3×10^5 cells. Three tubes were used per dilution and titres were calculated according to the method of Reed and Muench (1938).

RESULTS

Virus Isolation:-

Rate of virus isolation from sheep: Virus was isolated from seven out of the eight sheep inoculated intranasally (Group 1) and six out of the eight sheep inoculated intravenously (Group 2).

In group 1, virus was isolated from three sheep on day four, six sheep on day eight and seven sheep on day 12 and five sheep on day 16 post-inoculation (PI) (table 17). The rate of virus isolation varied with the days of sampling ($P < 0.001$) (Appendix table G). The best samples for virus isolation were those taken on day eight and 12 and the worst were those taken on day four and 16 ($P < 0.001$). The difference between the efficiency of isolation on day eight and 12 was not significant ($P > 0.300$).

One of the sheep in group 1 gave birth to a stillborn lamb three weeks PI and BVDV was isolated from the kidneys, heart and intestinal homogenates of the lamb.

In group 2, virus was isolated from five sheep on day four, six on day eight and one sheep on day 12 PI. Samples taken on day 16 and 20 PI were all negative (table 17).

Rate of virus isolation from samples: In group 1, five blood samples, 16 faecal samples and 15 nasal samples gave virus. All blood samples were negative on day four, 16 and 20, two were positive on day eight and three were positive on day 12 PI (table 18). Blood samples taken from four sheep were negative at all times.

Two nasal swabs were positive on day four, five swabs on day eight, five on day 12 and three on day 16 PI. Swabs from one sheep were negative throughout the experiment (table 18).

One faecal sample was positive on day four, five samples on day eight, six on day 12 and four on day 16 PI. No virus was isolated from the faeces of two sheep (table 18).

TABLE 17

RATE OF VIRUS ISOLATION FROM SHEEP INOCULATED INTRANASALLY (GROUP 1) AND SHEEP INOCULATED INTRAVENOUSLY.

Group	Route of Infection	Days post-inoculation					Total
		4	8	12	16	20	
1	intra-nasally	3	6	7	5	0	21
2	intra-venously	5	6	1	0	0	12
Total		8	12	8	5	0	33

TABLE 18

RATE OF VIRUS ISOLATION FROM SAMPLES COLLECTED FROM SHEEP INFECTED INTRANASALLY (GROUP 1)

Samples	Days PI					Total
	4	8	12	16	20	
Whole blood	0	2	3	0	0	5
Nasal swabs	2	5	5	3	0	15
Faecal swabs	1	5	6	4	0	16
Total	3	12	14	7	0	36

In group 2, twelve blood samples and one faecal sample gave virus (table 19). Five blood samples were positive on day four, six on day eight and one on day 12 PI. Only one faecal sample was positive on day eight PI (table 19).

TABLE 19

RATE OF VIRUS ISOLATION FROM SAMPLES COLLECTED FROM SHEEP INFECTED INTRAVENOUSLY (GROUP 2)

Samples	Days PI					Total
	4	8	12	16	20	
Whole blood	5	6	1	0	0	12
Nasal Swabs	0	0	0	0	0	0
Faecal Swabs	0	1	0	0	0	1
Total	5	7	1	0	0	13

Virus titres in the samples: In group 1, the virus content of the samples was so low that virus was only isolated after the second or third blind passage in cell cultures (Appendix table C). In group two the virus titre in blood ranged from a trace to $10^{3.5}$ $TCID_{50}$ per ml (table 20). The virus content of the faecal sample was so low that it required some blind passages before the virus was isolated.

TABLE 20

VIRUS TITRES IN BLOOD SAMPLES COLLECTED FROM SHEEP INOCULATED INTRAVENOUSLY (GROUP 2)

Day P1	Sheep No.							
	9	10	12	34	50	980	884	991
4	1.5*	1.2	1.1	3.5	Nil	Nil	Nil	Trace
8	2.4	1.8	2.8	1.9	0.3	Nil	Nil	0.7
12	Trace	Nil	Nil	Nil	Nil	Nil	Nil	Nil

* Given as $\log_{10}$ per ml of blood

Virus isolation in different cell cultures: In group 1, eleven samples gave virus in BEK, 25 samples in LT and 18 samples in CT cells (table 21).

TABLE 21

RATE OF VIRUS ISOLATION IN BEK, LT AND CT CELLS FROM SAMPLES TAKEN FROM SHEEP INOCULATED INTRANASALLY (GROUP 1)

Cells	Blood	Faeces	Nasal	Total
BEK	2	8	1	11
LT	4	10	11	25
CT	2	7	9	18
Total	8	25	21	54

In all 32 blood samples, 32 faecal samples and 32 nasal samples were collected and inoculated into three types of cell cultures. The difference in the rate of virus isolation

among the three types of samples was highly significant ($P < 0.010$) (Appendix table D). The difference between blood and faeces was highly significant; the difference between blood and nasal swabs was still significant but the difference between faeces and nasal swabs was not significant (Appendix Table E). The highest rate of virus isolation was achieved from faeces and the lowest rate from blood, nasal swabs being in-between.

The difference in the susceptibility of BEK, LT and CT cells to BVDV was significant ($P < 0.050$) (Appendix Table D). The difference between the rate of virus isolation in BEK and CT cells; CT and LT cells was not significant but the difference between BEK and LT cells was significant ($P < 0.020$) (Appendix Table F), LT cells giving the highest rate of isolation.

In group 2, ten samples gave virus in BEK, eleven gave virus in CT and 13 gave virus in LT cells (table 22).

TABLE 22

RATE OF VIRUS ISOLATION IN BEK, LT AND CT CELLS FROM SAMPLES COLLECTED FROM SHEEP INOCULATED INTRAVENOUSLY (GROUP 2).

Cells	Blood	Faeces	Nasal	Total
BEK	10	0	0	10
LT	12	1	0	13
CT	11	0	0	11
Total	33	1	0	34

Antigen Detection by Immunofluorescence:

The immunofluorescent test did not detect any further infections. Its advantage was that it always picked out infected cultures one passage before the development of CPE when samples examined came from group 1.

With the one positive faecal sample from group 2, all the coverslips examined on the third day post-inoculation showed specific BVDV fluorescence and when the tubes were reincubated, CPE developed in one tube 24 hours later.

Antibody Response in Sheep:

When the antibody response of sheep was measured by the microtitre neutralization technique, the titres progressively increased until they reached maxima ranging between $\log_{10}$ 1.2 to 2.7 at the sixth to eighth weeks P1. The earliest detectable antibody titres were observed in ten out of the 16 sheep at the second week P1 and this titre was $^{1}/2$ to $^{1}/4$ (table 23). Up to the third week the sheep inoculated by the intranasal route had significantly higher titres than those shown by the sheep inoculated intravenously ($P < 0.05$) (table 24). After the fourth week P1, the differences in titres among the two groups were not significant.

Contagion of BVD Among Sheep:

The two sheep kept with those inoculated by the intravenous route did not show any detectable antibody after six weeks of observation. One of the two sheep kept with those inoculated by intranasal instillation had a low titre

of antibody namely $^{1}/4$ per 100 $TCID_{50}$ of the NADL strain of BVDV but the second sheep did not show any response.

The four sheep inoculated intranasally with 10^{4} $TCID_{50}$ and 10^{3} $TCID_{50}$ had appreciable titres of antibody but those inoculated with 10^{2} $TCID_{50}$ or less, either had very low titres or were negative (table 25).

TABLE 23

ANTIBODY TITRES(x) IN BOTH GROUPS OF SHEEP INFECTED BY BVDV

Weeks Post-inoculation	Group 1 sheep								Group 2 sheep							
	691	676	784	790	851	852	902	903	9	12	10	30	50	980	884	991
1st Week	0	0	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0
2nd Week	0	0.6	0.6	0.6	0.3	0.3	0.6	0.3	0	0	0	0.3	0	0.2	0	0.6
3rd Week	0.6	1.0	1.9	1.2	0.9	0.9	1.3	0.8	0.3	0.9	0.2	1.2	0.6	0.6	0.4	0.9
4th Week	1.5	1.3	1.8	1.5	0.9	0.9	1.5	1.2	1.0	0.9	0.6	1.2	1.2	1.4	1.0	1.2
5th Week	1.5	1.5	1.8	1.5	0.1	0.9	1.6	1.7	1.2	1.2	1.0	1.8	1.8	1.7	1.3	1.5
6th Week	1.8	1.5	1.8	1.8	1.2	1.2	2.3	2.5	1.2	1.5	0.9	2.0	2.0	2.2	1.8	1.5
8th Week	1.8	1.8	2.7	1.8	1.2	1.1	2.3	2.7	1.8	1.5	1.1	2.1	Died	2.2	1.8	2.1
12th Week	1.8	1.8	2.7	1.8	1.2	1.2	2.3	2.7	1.8	1.5	1.2	2.0	-	2.1	1.8	2.3

(x) given as $\log_{10}$

TABLE 24

DIFFERENCES IN ANTIBODY RESPONSE BETWEEN THE TWO GROUPS OF SHEEP INOCULATED INTRANASALLY AND INTRAVENOUSLY

Weeks P1	GROUP 1			GROUP 2			t
	N	Mean	Standard deviation	N	Mean	Standard deviation	
1	8	0.04	0.11	8	0	-	-
2	8	0.41	0.22	8	0.14	0.22	2.454 *
3	8	1.08	0.40	8	0.64	0.34	2.369 *
4	8	1.33	0.32	8	1.06	0.24	1.915
5	8	1.45	0.30	8	1.44	0.31	0.066
6	8	1.76	0.47	8	1.64	0.44	0.527
8	8	1.93	0.60	7	1.80	0.40	0.485
12	8	1.94	0.60	7	1.81	0.37	0.794

N = number of animals used

* significant difference between the two groups ($P < 0.05$)

TABLE 25

TITRES OF BVD ANTIBODY IN SHEEP INOCULATED BY DIFFERENT DOSES OF BVDV (NADL STRAIN)

Dose used	Neutralizing antibody			
	1	2	3	4
10^4 $TCID_{50}$	2.1*	1.9	2.0	2.0
10^3	0.9	1.6	1.9	1.6
10^2	0.3	0	0.6	0
10	0	0	0	0
1	0	0	0	0

* = given as $\log_{10}$ per 0.05 ml.

DISCUSSION

BVDV was regularly isolated from nasal and oral swabs collected from naturally infected cattle as long as lesions were present (Van Bekkum and Straver, 1966).

The sheep used in this experiment exhibited no lesions, nevertheless BVDV was isolated from their faeces and nasal secretions up to the 16th day after intranasal exposure. The best samples for virus isolation were the faeces, followed by the nasal secretions and the worst sample was blood. Pardo (1977), using calves, also found that nasal samples gave better isolation results than buffy coats.

In the sheep inoculated intravenously, the virus was isolated from the faeces of one sheep and was never isolated from the nasal secretions. It was regularly isolated from the blood of some sheep until the eighth day after inoculation but on the 12th day it was only isolated from one sheep. Likewise, Van Bekkum and Straver (1966) reported that they isolated the virus from the blood of calves up to 10 days after inoculating them, but they could not isolate the virus from faeces, urine, and nasal or oral swabs.

The efficiency with which virus was isolated also depended on the type of cell culture used, LT cells giving the best results, followed by CT and lastly BEK cells. The high rate of virus isolation in LT cells was probably attributable to the passage of the virus in LT cells before it was inoculated into sheep. Immunofluorescence reduced the time for confirming BVDV isolation.

Both groups of sheep produced serum neutralizing antibodies which reached similar maxima by about the sixth week after inoculation. The antibody response of the sheep inoculated by the intranasal route was however significantly higher in the first three weeks.

CHAPTER 5

STUDIES ON THE RELATIONSHIPS AMONG BOVINE VIRUS DIARRHOEA, BORDER DISEASE AND RUBELLA VIRUSES

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INTRODUCTION

Even a mild infection with BVDV, BDV or Rubella virus (RV) during pregnancy crosses the placenta and causes abortion or foetal dysmorphogenesis (Ward et al., 1969, Barlow and Dickinson, 1965, Siegel and Greenberg, 1960). Immunoglobulins of foetal origin have been detected in the sera of calves from BVDV infected cows (Kniazeff et al., 1967), in the sera of lambs from BDV infected ewes (Gardiner, 1967) and in the sera of children from RV infected mothers (Soothill et al., 1966).

Acland et al., (1972) and Hamilton and Timony (1972) implicated BVDV in the aetiology of BD when they found that sera from BD infected animals reacted with BVDV. Huck et al., (1975) demonstrated fluorescent, complement fixing and neutralizing antibodies to BVDV in BD sera and Hadjisavvas et al., (1975) observed interference of BDV with BVDV which was neutralized with BVD antiserum. Harkness et al., (1977) reported that tissue cultures infected with BDV showed specific fluorescence with a conjugated hog cholera antiserum; a property also shared by BVDV (Meyling et al., 1963). Morphological similarities between BVDV and RV were reported (Horzinek et al., 1971).

This investigation was intended to explore the extent of the relationship between BVDV and BDV and to test the immunological relationship between RV and BVDV.

MATERIALS AND METHODS

Virus strains

BVDV:- Two strains were used for the hyperimmunization of sheep: the cytopathic NADL strain and a non-cytopathic strain isolated from an infected BEK processed for tissue culture. The latter strain was designated NCPBVD2 and was found to propagate in BEK, CT and LT cells. Two CP strains (CT275041 and CT3 73758) were obtained from the Central Veterinary Laboratories, Weybridge.

BDV:- Two strains were used for hyperimmunizing sheep. One strain was received as a suspension of brain and spinal cord from a border disease-infected lamb (Vantsis et al., 1978). A cytopathic virus strain was isolated from the suspension in lamb testis cells and purified by the selection of a single plaque on three successive passages and designated (BDM). The second strain, a noncytopathic strain originally isolated in CT cells was obtained from Dr. Harkness of the Central Veterinary Laboratories, Weybridge; it was designated (BDW).

RV:- A strain of rubella virus which causes degeneration of RK-13 cell monolayers, was obtained from Dr. Inglis, City Hospital, Edinburgh. A rubella haemagglutination antigen and its negative control were obtained commercially[1].

Cell Cultures:

RK-13 cell line cultures were propagated in 300 ml bottles

1 Wellcome Laboratories, Beckenham.

in medium 199 supplemented with 10 percent FCS. Cells were then split into test-tubes and maintained in medium 199 supplemented with 5 percent FCS.

Interference Between BVDV and RV:-

Ten tubes of RK-13 cultures were inoculated with 10^{-1} dilution of the NADL strain (10^{3} $TC1D_{50}$ per 0.1 ml) and ten tubes were left as controls. Five days later, the cultures were challenged with 0.1 ml of 10^{2} and 10^{3} $TC1D_{50}$ of RV.

A similar number of BEK tube cultures were inoculated with a 10^{-1} dilution of RV (10^{2} $TC1D_{50}$ per 0.1 ml). Five days later they were challenged with 0.1 ml of 10^{2}, 10^{3} and 10^{4} $TC1D_{50}$ of the NADL strain and all tubes were observed for CPE for seven days.

Antisera:

A rubella haemagglutination inhibiting antiserum with $^{1}/_{32}$ titre and negative serum control were obtained from Dr. Inglis, City Hospital, Edinburgh. Antisera against the four strains of BVDV and BDV were prepared in sheep which were seronegative at a 1/2 dilution of serum against 10^{2} $TC1D_{50}$ of the NADL strain of BVDV. The viruses were propagated in LT cells maintained in MEME supplemented with 5 percent TPB and 2 percent sheep serum. Antiserum for each virus was prepared by the immunization of two sheep. Each sheep was infected by the intranasal instillation of 5 ml of the clarified fifth passage of the virus. Four weeks later each sheep was

inoculated intravenously on two successive weeks with 5 ml of the homologous virus strain. Three weeks after the last injection sheep were bled and the serum separated and stored at -20°C. Equal volumes of the sera from sheep immunized by the same virus were pooled.

Testing of Sera:

The microtitre serum neutralization test was used with the cytopathic viruses and the standard tube interference test was used with the NCP strain of BVDV. To determine the immunological relationship between BVDV and RV, antiserum for RV and the negative control serum were diluted ten-fold and tested by the microtitre neutralization technique against ten-fold dilutions of the NADL strain of BVDV. Two-fold dilutions of BVD antiserum prepared in a calf were tested by the tube serum neutralization test against 10^2 $TC1D_{50}$ of RV.

Two-fold dilutions of the calf BVD antisera, the calf preinoculation serum, BVD antiserum prepared in a gnotobiotic calf at Compton and a convalescent serum from a field case with a neutralization titre of 1:512 were tested against RV haemagglutinins.

To determine the immunological relationship between BVDV and BDV, the antisera to the NADL and NCPBVD2 strains of BVDV and the antisera to BDM and BDW were tested against 10^2 TCID of the NADL strain of BVDV and 10^2 $TC1D_{50}$ of BDM strain of BDV. Sera were diluted 1/40 and their neutralization indices against the NADL strain of BVDV and the BDM strain of BDV calculated. Antisera were also

diluted 1/100 and their neutralization indices to four different strains of BVDV determined.

Interference neutralization test: The fifth passage of NCPBVD2 in BEK cells was clarified and ten-fold dilutions of the virus prepared and mixed with equal volumes of a 10^{-2} dilution of the serum to be tested. After one hour incubation at 37°C 0.2 ml of each dilution were inoculated into three tubes of BEK cells. Tubes were then incubated for 90 minutes at 37°C to allow for adsorption before 1 ml of maintenance medium was added. Three tubes were inoculated with 0.2 ml of 1/200 dilution of each antiserum as serum controls. The control serum was similarly treated.

Five days later, the medium was decanted and the tubes rinsed three times with PBS prewarmed to 37°C. Each tube was then challenged with 10^2 $TCID_{50}$ of the NADL strain in 0.1 ml. and the tubes were observed until complete CPE developed in control tubes inoculated with test sera only. Tubes showing CPE were taken as those in which the NCP virus strain had been neutralized; titres were calculated according to the method of Reed and Muench (1938).

Haemagglutination inhibition test:

Red blood cells: One day-old chicks were bled from the heart into syringes containing Alsever's solution. The collected cells were washed three times in dextrose-gelatin-veronal buffer (DGV) pH 7.3[1] and packed in a graduated centrifuge tube by centrifugation at 1500g for 10

1 Gibco Bio-cult

minutes. A 10 percent suspension in DGV was then prepared and stored at 4°C until required.

A 50 percent suspension was used for adsorbing non-specific agglutinins in the test sera and 0.2 percent suspension was used for the test.

Adult chicken cells similarly treated were found to give similar results.

Titration of rubella haemagglutinins: Two-fold dilutions of the antigen in 0.025 ml volumes were prepared in the wells of a microtitre haemagglutination plate. Equal volumes of Hepes-saline-albumin-gelatin (HSAG) diluent pH 6.2[1] were dropped into each well containing the haemagglutinin dilutions. Two wells, each containing 0.05 ml of HSAG were prepared as cell controls. Then 0.05 ml of a 0.2 percent erythrocytes suspension were added to each well and the plate gently shaken, covered and left at 4°C for 60 minutes. Thereafter the cells were allowed to settle at room temperature for 15-30 minutes. One haemagglutinin unit was taken as the highest dilution giving complete agglutination and four units were used in the test.

Testing of Sera: Calf sera were treated by two methods for the removal of nonspecific haemagglutination inhibitors, namely heparin-manganous chloride and neutral kaolin (Lennetle and Schmidt, 1969). Sera were tested by the microtitre technique (Lennette and Schmidt, 1969) except that HSAG diluent was used instead of DGV.

1 Gibco Bio-cult

The serum titres were recorded as the highest dilution which gave complete inhibition of agglutination.

RESULTS

Relationship Between BVDV and RV

The inoculation of RK-13 monolayers with the NADL strain of BVDV did not result in cytopathic changes. Coverslips treated with fluorescein conjugated BVD antiserum fluoresced irrespective of whether they were inoculated or not. When these cultures were challenged with RV, CPE developed in tubes previously inoculated with BVDV as well as in the controls. RV did not show CPE in BEK cells but the cultures resisted challenge by the NADL strain of BVDV up to a titre of 10^4 $TCID_{50}$ per 0.1 ml.

Bovine sera treated with heparin-manganous chloride (HMC) for the removal of non-specific inhibitors, inhibited the agglutination of chicken RBC by rubella haemagglutinins (Table 26). Treatment of the same sera with kaolin removed the haemagglutinin inhibitory effect of the sera indicating that the effect was non-specific.

TABLE 26

HAEMAGGLUTINATION INHIBITION TITRES WITH RUBELLA AND BVD ANTISERA

Sera	Treated with HMC	Treated with Kaolin
Positive rubella serum control	32*	32
Negative rubella serum control	Nil	Nil
Calf preinoculation serum	64	4
Calf BVD antiserum	64	Nil
Gnotobiotic calf BVD antiserum	8	Nil
Serum from BVD field case	32	Nil

* Expressed as the reciprocal of the end point dilution.

Relationship Between BVDV and BDV:

The neutralization titres of the two BVDV antisera against BVDV were $10^{2.4}$ and $10^{3.0}$ per 0.05 ml; the titres of the BDV sera against BVDV overlapped being $10^{1.9}$ and $10^{2.7}$, BDW antiserum giving the lowest titre (table 27).

With BDV, the neutralization titre of antiserum to the NADL strain of BVDV was the highest at $10^{3.2}$. The neutralization titres of antisera to the NCP strain of BVDV and BDM strain of BDV were similar at $10^{2.7}$. Antiserum to BDW strain of BDV gave the lowest titre, $10^{2.1}$ (table 27).

With the NADL strain of BVDV as antigen, and 1/40 dilution of antisera, the neutralization indices of BVDV and BDV strains overlapped being 3.5 and > 4.7 with BVDV antisera and 2.2 and 4.2 with the two BDV antisera. With BDM strain of BDV as antigen, the neutralization indices of both BVDV antisera and BDM antisera were 3.0. The neutralization index of BDW strain of BDV was 2.5 (table 27).

The neutralization indices against four different strains of BVDV, using $^{1}/100$ dilution of antisera varied with each serum (table 28). These indices are averages of three tests done at different times. The antiserum to the NCP strain of BVD gave the highest index with the homologous virus and the lowest index with CT3 strain. The antisera to the NADL strain gave similar indices with the homologous virus and with the CT2 strain. It did not reduce the titre of the CT3 strain.

The antisera to BDW of BDV gave the highest index with the NCP strain and did not reduce the titre of the CP NADL strain.

The BDM strain gave the highest index with the NADL strain and the lowest index with CT3 strain.

The neutralization index of the NADL antiserum against CT3 was zero whereas the two BDV strains antisera still reduced the titre of this strain of BVDV.

TABLE 27

NEUTRALIZATION TITRES AND INDICES OF ANTISERA TO BVDV AND BDV AGAINST HOMOLOGOUS AND HETEROLOGOUS VIRUSES

Antisera, $^{1}/_{40}$	Neutralization titres(x)		Neutralization indices (*)	
	NADL	BDM	NADL	BDM
NCP-BVD$_2$	2.4	2.7	3.5	3.0
NADLBVD	3.0	3.2	> 4.7	3.0
BDW	1.9	2.1	2.2	2.5
BDM	2.7	2.7	4.2	3.0

(x) Expressed as the negative logarithm of the 50 percent dilution

(*) Expressed as the logarithm of virus neutralized

TABLE 28

NEUTRALIZATION INDICES OF BVDV AND BDV ANTISERA AGAINST FOUR STRAINS OF BVDV

Antisera,[1]/100	Neutralization indices*			
	NADL	(CT2) 75041	(CT3) 73758	NCP - BVD_2
NCP-BVD2	1.5	1.2	1.0	1.9
NADL-BVD	2.5	2.5	00	1.2
BDW	0.0	0.8	0.4	1.5
BDM	1.5	1.0	0.3	1.6

* Average of three tests done at different times and given as the logarithum of virus neutralized

DISCUSSION

Cross-neutralization studies on a RV strain and the NADL strain of BVDV indicated that there was no serological relationship between the two viruses although RV interfered with the propagation of the CP strain of BVDV in BEK cell cultures. This finding indicates that unrelated viruses may interfere with the propagation of BVDV. Consequently, when the interference test is used for detecting NCP strains of BVDV, specific neutralization of interference must always be carried out for confirming the presence of NCP strains of BVDV in cell cultures.

The cross-neutralization patterns obtained between BVDV and BDV strains (Table 27) indicated that some strains of the heterologous virus might be more closely related than strains from the homologous virus. Antiserum to BDM strain of BDV showed a higher titre with the NADL strain of BVDV than the antiserum to a NCP strain of BVDV (Table 27). Also the two BDV antisera had low neutralization indices with CT3 strain of BVDV while the neutralization index of the NADL antiserum with the same BVDV strain was zero (Table 28). The neutralization indices obtained against the different strains of BVDV (Table 28) suggested that although the different BVDV strains are serologically closely related, each virus strain differed serologically from the others. The NADL antiserum with a neutralization index of 2.5 with the homologous virus and a similar neutralization index with CT2 strain indicated that the two strains were homologous, but

BDW antiserum at its 1/100 dilution had no effect on the NADL strain while it showed a neutralization index of 0.8 with the CT2 strain.

In summary, the BVDV strains were serologically closely related, and they were closely related to the BDV strains investigated.

CHAPTER 6 Page

OVINE SERUM AS A SUPPLEMENT FOR BOVINE EMBRYONIC KIDNEY CELLS

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INTRODUCTION

When sheep and lamb sera were used in the maintenance medium for BEK cells, the development of monolayers was apparently enhanced. Also during the preliminary studies on the microtitre serum neutralization test, sheep serum was observed to support better monolayer cover in the cups of the microtitre plates when it was used in comparison with FCS. These unexpected observations initiated the study of the possibility of growing BEK cells in SS or LS as ovine sera are less likely to contain BVD antibodies (French and Snowdon, 1964; St. George *et al.*, 1967, St. George, 1971).

MATERIALS AND METHODS

Sera:

Mycoplasma-screened, heat inactivated LS which had been used for the propagation of LT cells was compared with FCS and HS (Gibco Bio-Cult, Glasgow) both of which were also heat inactivated.

Propagation of Cell Cultures:

Stored BEK cells which were previously tested for contamination with BVDV were resuscitated and suspended in the growth medium at the rate of 3.5×10^5 cells per ml. Cultures were propagated in 50 ml plastic tissue culture bottles and each bottle was seeded with 5 ml of the suspension.

Growth medium:

MEME was supplemented with 10 percent serum, 10 percent TPB, vitamins, NEA and antibiotics.

Evaluation of Cell Growth:

After 18 hours of incubation, 24 bottles were randomly selected, eight for each serum. The bottles were observed under the microscope and then the attached cells were harvested with STV, centrifuged at 200g for 10 minutes and the STV discarded. Cells from each bottle were resuspended in HBSS and counted electronically.

The growth medium in the remaining bottles was changed and the cultures allowed to proceed to full monolayers. Three days later cultures were again observed under the microscope

and cells resuspended and counted electronically.

Testing Cultures Susceptibility to BVDV:

The third passage of cell cultures were propagated on coverslips in Leighton tubes and monolayers inoculated with 10^2 $TCID_{50}$ of the NADL strain of BVDV. Cultures were observed for CPE and coverslips from infected and control tubes examined by the direct immunofluorescence technique after 24 hours of incubation.

RESULTS

Cell Attachment:

Uniform distribution of attached cells was observed with both FCS and sheep serum. In both cases, most of the cells were fibroblastic. With horse serum, the attached cells were distributed along the surface in irregular clumps and were small and round. The mean of the attached cells counts were $(1.07 \pm 0.19) \times 10^5$ cells per ml for HS, $(1.33 \pm 0.23) \times 10^5$ cells per ml for SS and $(1.7 \pm 0.3) \times 10^5$ cells per ml for FCS (table 29). The differences among the sera were highly significant (Probability < 0.010). Using Dunan's Multiple Range Test, the sera fell into two overlapping subsets, namely FCS + SS and SS + HS. The FCS resulted in the attachment of 49 percent of the viable cell count, the sheep serum resulted in 38 percent and the horse serum only 31 percent.

Monolayer Formation:

FCS and SS serum promoted very good monolayer covers. The total cell counts in the monolayers were $(4.72 \pm 0.31) \times 10^5$ cells per ml for HS, $(7.1 \pm 0.74) \times 10^5$ cells per ml for SS and $(6.96 \pm 1.35) \times 10^5$ cells per ml for FCS (table 30). The counts with SS were as high as those shown by FCS ($P > 0.80$). The counts with HS showed significant difference from the counts with FCS ($P < 0.010$).

TABLE 29.

NUMBER OF CELLSATTACHED IN EIGHT REPLICATES FOR EACH SERUM

Serum	1	2	3	4	5	6	7	8	Mean $\pm$ standard deviation
Horse serum	0.76*	1.19	1.24	1.36	1.07	0.87	1.02	1.08	1.07 $\pm$ 0.19
Sheep serum	0.92	1.25	1.11	1.56	1.50	1.43	1.30	1.59	1.33 $\pm$ 0.23
Foetal calf serum	1.55	1.44	1.79	2.33	1.95	1.60	1.65	1.48	1.72 $\pm$ 0.3

* x 10^5 cells/ml of the original volume

TABLE 30

NUMBER OF CELLS IN THE MONOLAYERS AT THE FIFTH DAY POST SEEDING WITH THE DIFFERENT SERA TRIED IN FIVE REPLICA FOR EACH SERUM

Serum	1	2	3	4	5	Mean + standard deviation	Percentage viability
Horse serum	4.70*	4.45	4.46	4.80	5.20	4.72 ± 0.31	78.57
Sheep serum	7.36	8.10	7.28	6.20	6.56	7.10 ± 0.74	81.55
Foetal calf serum	7.76	5.64	9.11	7.22	6.06	6.96 ± 1.35	92.83

* $\times 10^5$ cells/ml of the original volume

DISCUSSION

Sheep serum used as a supplement for growing BEK cells was found to be as efficient as FCS. It helped the formation of the uniform monolayers which were susceptible to BVDV to the same degree as cells propagated in the presence of FCS.

When coverslips were tested by the direct fluorescence test, specific BVDV fluorescence was observed in some of the control uninoculated tubes. This was surprising as this kidney was previously tested and found to be negative for BVDV. Contamination of the ovine serum was suspected but this was excluded when the same serum was used to grow cells from another known negative kidney. Repeated tests on the first kidney proved that it was consistently showing some degree of fluorescence.

At this stage, it was suspected that the adventitious virus contamination had probably been concealed by a very low titre of antibody in the FCS used earlier for the propagation of cultures from this kidney. Serum aliquots which were previously collected in bijous and which were negative for BVDV antibody at 1/4 dilution against 100 $TC1D_{50}$ of the NADL strain of BVDV were tested for serum neutralizing antibodies in the microtitre serum neutralization test against 50 $TC1D_{50}$ of the CT3 73758 strain of BVDV. Their neutralization indices at their $^{1}/2$ dilution were also determined against 10-fold dilutions of the NADL strain of BVDV (Appendix table H). Five of the seven sera tested, had some titre of neutralizing

antibody against the CT3 strain and six of the seven reduced the titre of the NADL strain of BVDV.

When the sera were run in the Electrophoresis Densitometer[1] one of them contained gamma globulins. All the sera contained beta and alpha globulins which were mainly in the alph 1 fraction.

These findings indicated that FCS might contain low titres of antibody which could conceal contaminated kidneys. This low titre of antibody was not detected because only one strain of the virus was used. Other strains of BVDV might have detected the antibody.

Ovine serum proved to be a safer supplement for growing LT cells intended for BVD studies (Seibold and Dougherty, 1967), but it has not yet been regularly used for the propagation of BEK cells.

1 Millipore Corporation, U.S.A.

CHAPTER 7

GENERAL DISCUSSION

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INTRODUCTION

The necessity for a prompt diagnosis of BVD arises from its clinical simulation of other diseases commonly responsible for disasterous and devastating epidemics such as rinderpest and foot and mouth disease. BVD has also been confused with less serious viral diseases such as malignant catarrhal fever, infectious bovine rhinotracheitis and bovine papular stomatitis, many bacterial and rickettsial diseases such as salmonellosis, pasteurellosis and bovine petechial fever, protozoal infections like east coast fever and the effects of poisonous plants and minerals (Olafson et al., 1946; Kahrs, 1971; Hull, 1972).

Confirmation of a presumptive diagnosis of BVD is fraught with difficulty. The quickest and least controversial method is isolation of the virus in cell culture and identification of its presence by the detection of specific antigens by immunofluorescence. My studies confirmed however, that the risk of contamination of cultures and sera used for cultures with adventitious viruses and antibodies reported by Fogh et al., (1971) and Molander et al., (1972) is real. Confirmation by detecting a rise in specific antibodies is, in most cases, confused by the ubiquitous presence of the virus. Nevertheless, the detection of BVD antibody in the sera of cattle that were seronegative three weeks earlier is convincing evidence that BVD has occurred (Kahrs, 1971). The time that elapses before the collection of the second serum sample renders the method inefficient and

nullifies control of serious epidemics with similar clinical features. Some workers recommended the testing of a single serum from a clinically infected animal and suggested that a negative serum be considered as a positive diagnosis for BVD (Borgen, 1963; Bürki and German, 1964; Snowdon, 1973), but this method does not exclude the presence of a serious clinically similar disease.

RINDERPEST AND BVD

The close similarity between the clinical and pathological features of BVD and RP stimulated Walker and Olafson (1947) to find out if there was any immunological relationship between the two viruses. In fact the two diseases are so similar that in countries where RP has been eradicated and where BVD is a menace, an outbreak of RP will not be suspected early enough to curb the spread of RP if efficient diagnostic methods are not available. The classical epidemiological features of RP are based on episodes in virgin-soil epidemics. In countries where RP has long been endemic, the common form of infection is the subclinical case in an immature animal (Scott, 1967). Moreover relatively avirulent strains of RP have evolved naturally (Wilde and Scott, 1961). Consequently Kahrs (1971) wrote, "Rinderpest must always be considered when diagnosising BVD".

In countries where rinderpest prevails, it is also necessary to be able to differentiate the two diseases as early as possible, because efficient control of RP involves

an embargo on animal movement and trade, a process which many developing countries can ill afford. A provisional diagnosis of rinderpest based on the clinical features, morbid anatomical changes and epidemiological behaviour was considered enough to justify vigorous control measures until confirmation of the disease was achieved by the isolation and serologic identification of the virus, the demonstration of specific antigens or the detection of the development of antibodies (Scott, 1964).

Vaccination of cattle against RP has proved to be efficient and has altered the epidemiological picture. Provost (1972) however, drew attention to a new diagnostic problem when he found that RP vaccinated cattle as well as calves passively protected by injesting colostrum could harbour virulent virus in their nasal passages and transmit it to susceptible cattle. Although this is of minor epidemeological significance, if such animals show the clinical signs of mucosal disease, they will be confirmed as RP. Also fresh diagnostic problems have arisen with the emergence and recognition of peste des petits ruminants (PPR) virus as being distinct from but serologically related to rinderpest virus (Hamdy, et al., 1975).

My studies indicated that in countries where RP is endemic, it will be easier to confirm a diagnosis of RP than a diagnosis of BVD. A more serious problem arises in countries where RP is not endemic; if RP occurs in such countries, the first cases will be dismissed as BVD because confirmation of a provisional diagnosis of BVD is seldom

attempted. These studies were intended to rectify deficiency in the protocol for diagnosing BVD by defining the hazards, particularly the hazards of interpretation of the results of virus and antigen detection. These hazards are either associated with tissues used in cell cultures or the serum supplements for growing and maintaining the cells.

CELL CULTURE HAZARDS

Cell Type Problems:

The first propagation of BVD virus was in bovine embryonic kidney (BEK) and foetal muscle cell cultures by Lee and Gillespie (1957). This was followed by the propagation of the virus in bovine calf testes (Gratzek, 1961), bovine embryonic trachea (Pritchard, 1963), bovine buffy coats (Gutekunst and Malmquist, 1964), lamb testes (Seibold and Dougherty, 1967), Madin-Darby bovine kidney cell line (Marcus and Moll, 1968), Hamster kidney, human ERK-1 and pig kidney tissues PK-15 (Fernelius et al., 1969) bovine foetal spleen and bovine foetal lung (Scott et al., 1972), bovine endocardial cells (Archbald et al., 1973), bovine turbinate cells (McClurkin et al., 1974) and BEK cell line-AU-BEK (Cancellotti and Turilli, 1975).

It has always been difficult to find a consistently susceptible cell line probably due to contamination with adventitious viruses resulting from the bovine sera used for their propagation. In my studies rabbit kidney (RK-13), pig kidney

(PK-15), Madin-Darby bovine kidney (MD-BK) and Vero cell lines cultured for the propagation of BVDV were all found to show positive BVDV fluorescence in an indirect fluorescence test. Moreover I failed to propagate the NADL strain in these cell cultures.

McFerran et al., (1972) concluded that primary kidney cell cultures derived from the same species as that of the specimen being tested were necessary for the efficient detection of virus for diagnostic purposes. Consequently BEK cells which were not infected by interfering viruses should be the best system for the isolation of bovine viruses. I also found that they gave clearer cut results in titrations of BVDV when compared with other systems.

Nevertheless there are many difficulties associated with their propagation, and maintenance in addition to the fact that they are not easily available because pregnant cows are not commonly slaughtered.

Problems of Cell Resistance:

Some workers claimed that BEK cells were not as sensitive as other cells (Malmquist 1968) and that their susceptibility was not uniform (Mills and Luginbuhl, 1965). Intrauterine infection of the foetus by interfering non-cytopathic BVDV strains can result in the infection of its kidneys (Gillespie et al., 1967). Such contaminated kidneys invalidated or confused efforts to isolate BVDV and resulted in 2-3 log drop in virus titre (Brown et al., 1968). Four $\log_{10}$ difference was observed between two kidneys (E and F table 3).

The incidence of BVD infection in normal bovine kidneys used for cell cultures varied from 2.7 percent (Ruckerbauer et al., 1971) to 30 percent (Schell et al., 1972). My data (table 2) indicated an infection rate of 40 percent; a rate that agrees with the serological evidence that more than 70 percent of Scottish cattle were infected (Snodgrass, 1977 - personal communication).

As a precautionary measure against the presence of latent adventitions viruses, workers were advised to screen cells by the interference and fluorescence tests (Classic and Fernelius, 1970). Other workers reported that animal inoculation and the interference test using the plaque reduction technique were more efficient in detecting adventitions viruses present in very low titres (Heuschele, 1975, Nuttal et al., 1977). My findings (table 2) confirmed that more than one test was necessary to ensure freedom from adventitious viruses.

In addition to interfering viruses, other contaminants such as mycoplasma have interfered with the replication of viruses (Romano and Brancato, 1970), a problem which was not encountered in my studies.

Some strains of BVDV do not show CPE in BEK cell cultures. These are the so called non-cytopathic strains (Gillespie et al., 1967). Their detection requires further tests such as interference (Gillespie et al., 1962), END (Inaba et al., 1963), Fluorescence (Fernelius, 1964) and inoculation of animals and detection of resulting antibody (Heuschele, 1975). These strains are not known to show CPE in any other cells and this adds to the difficulties of BVD diagnosis.

Problems of Cell Degeneration:

Degenerative changes simulating CPE can develop in uninfected control cultures (Hermodsson and Dinter, 1962). Mills and Luginbuhl (1965) suspected this phenomenon to result from overcrowding of cells. Wallis and Melnick (1969) postulated that the toxicity of some sera might be due to the low concentration of anti-enzyme components and Honn et al., (1975) showed that some serum batches contained toxic levels of substances like urea and creatine.

Contamination of cultures and/or sera is another source of degenerative effects. Contamination of cultures with yeast and bacteria or their L-forms resulted in cytopathogenic changes without showing any apparent turbidity when antibiotics were used in tissue culture media (Coriel, 1962; Ludovici and Christian, 1969). The presence of mycoplasma has been shown to cause a wide variety of cytological, immunological, genetical, biochemical and other changes in cell cultures (Fogh et al., 1971). Even pleuro-pneumonia-like organisms (PPLO) which are not normally cytopathogenic convert when they are passaged in tissue culture (Eaton, 1962). The source of tissue culture contamination with PPLO seems to be the conversion of bacterial contaminants to stable L-forms by antibiotics commonly used to prevent bacterial contamination (Holmgren and Campbell, 1960). Barile et al., (1962) found that 72 percent of cell cultures grown in media containing antibiotics were contaminated with PPLO as compared to 7 percent of those cultured in antibiotic-free media. Some of these

PPLO were totally dependant on the presence of live cells for their multiplication (Eaton, 1962).

Contamination of cell cultures with cytopathic BVD virus strains is another source of unexpected degeneration of controls. In one of the bovine embryonic kidneys I collected degenerative changes were observed at the third passage level (table 2). The cultures showed specific BVDV fluorescence in an indirect fluorescence test. The CPE was also transferable to BVDV-susceptible kidney cell cultures.

Cytopathogenic BVDV was isolated from aborted foetuses (Scott et al., 1972) as well as from BEK cell cultures (Ruckerbauer et al., 1971). Sattar et al., (1965) isolated a bovine herpesvirus which they identified as infectious bovine rhinotracheitis virus (IBRV) from the thoracic fluids of six foetuses and Belak and Palfi (1974) identified a herpes virus in BEK cell cultures which showed spontaneous degeneration. Parainfluenza-3 virus was also isolated from the thoracic fluid and lungs (Sattar et al., 1965) as well as from kidneys of bovine foetuses (Swift and Kennedy, 1972). Crandell et al., (1978) isolated a parainfluenza-2 virus strain from BEK cell cultures which they attributed to cross contamination from monkey kidney cell cultures.

Kniazeff (1968) summarised the properties that favour virus contamination of cell cultures. Firstly, there had to be a high incidence of infection in the host. Secondly, the infection had to be systemic and so promoting dissimination to tissues commonly used in cell cultures. Thirdly, the virus

had to persist in the host long enough to make isolation possible. Fourthly, it induced silent or mild disease and fifthly, it replicated and survived in cell cultures.

Viruses which satisfy all or most of these conditions include advenovirus, herpesvirus, parainfluenza virus, at least one enterovirus,BVDV and reovirus.

SERUM HAZARDS

Essentiality of Serum:

The use of bovine serum as a supplement for cell cultures has always been very difficult to avoid. There are many reasons. For example Wallis and Melnick (1969) and Temin et al., (1972) showed that serum and serum albumin respectively protected cells by their capacity to trap toxic substances such as lytic enzymes released by the cells in the culture media. Moreover, serum appears to contain a growth promoting factor which precipitated out with the gamma-globulin fraction by alcohol or ammonium sulphate (Todaro et al., 1967; Jainchill and Todaro, 1970). This growth promoting factor migrated with the gamma-globulin fraction through Sephadex G-200. Fractionated sera lost their cell growth stimulating capacity which was restored when the precipitated factor was redissolved (Jainchill and Todaro 1970).

The cholesterol content of the serum was reported to be an important cell attaching factor and was therefore of considerable importance in propagating cell culture monolayers (Boone et al., 1972). In my studies although the different

media used for growing BEK cells had different efficiencies in promoting monolayer formation, their effects on the number of cells attached were not significantly different (table 11). In contrast, although the FCS, HS and LS were used at the same concentration, there were significant differences between them in promoting attachment of cells (table 29).

Serum was shown to trigger the synthesis of DNA by cultured cells, an effect which was also potentiated by the addition of insulin to the medium (Clark et al., 1970; Dulbecco, 1970).

In spite of its usefulness, serum is the source of many problems in cell cultures when used for virus research.

Problems of Chemical Variation:

Serum is an extremely variable fluid. Different serum lots have been shown to vary in their chemical parameters and biological effects on cultured cells (Boone et al., 1972; Honn et al., 1975). These variations depended on the species, the age, the physical and the physiological status of the animal from which the serum was collected as well as the efficiency of the clotting of the blood and the separation of the serum.

The growth promoting activity of different lots of foetal calf serum (FCS) differed significantly when they were employed in growing human foetal lung cells (Boone et al., 1972). Neuman and Tytell, (1960); Boone et al., (1972) suggested that prevention of leucocytolysis was important when sera were

collected for growing tissue culture because proteases and other hydrolases released in the serum from leukocytes lysosomes could act on serum components to lower their nutritional value or to produce toxic products such as free fatty acids, the high content of which has inhibitory effect on cell growth. The extent of bacterial contamination in the serum before sterilization is another factor which has been suggested to result in the drop in growth supporting capacity of the serum, although this has been denied by Chu et al., (1973).

Boone and his colleagues (1972) reported that the protein content of FCS varied appreciably. For example they observed that foetal calf sera could have a protein content as low as 0.87 mg/100 ml and that such serum had a very poor cell growth supporting capacity. Boone et al., (1972) and Honn et al., (1975) observed that other constituents of serum also showed considerable variability. It is therefore not surprising that results of cell propagation vary with different lots of serum.

Problems With Anti-Viral Substances:

Sera from calves, horses and man are all known to have antiviral substances (Klein, 1958; Klein et al., 1959; Zilka et al., 1968). The antiviral activities of these substances are due to three different factors (Klein, 1958). Firstly, heat labile inhibitors that can be destroyed by the inactivation of the serum at 60°C for 30 minutes e.g. properdin. Secondly, heat stable inhibitors that are not globulins. These non-antibody virus inhibitors are not destroyed by heat

but can be absorbed by materials normally used in serology such as kaolin (Stewart et al., 1967). While I was testing the effect of BVD antisera on rubella haemagglutinin, I used sodium heparin and manganous chloride which are normally employed in the removal of rubella haemagglutination non-specific inhibitors but they failed to remove the non-specific inhibitors in the bovine serum (table 26). Thirdly, inhibitors that are globulins. These have been described in human, bovine, monkey, horse and rabbit sera and their activity can sometimes be abolished by 2-Mecraploethanol or simply by heating the serum at 70-80^{o}C for 15 minutes (Zilka et al., (1968). Inhibitors in this group are the main source of difficulties in BVD studies. Neutralizing antibodies to the virus are widely distributed in nature as reported in the different serological surveys. Borgen (1963a) reported 91 percent infection rate in a group of young animals. Bögel and Neuman (1964) reported that 72 percent of the cattle they tested had neutralizing antibodies to BVDV and St. George et al., (1967) reported that 70 percent of the bovine herds had neutralizing antibodies and that more than 60 percent of the animals were reactors. Sheep, goats and pigs, though to a lesser degree, had serum neutralizing antibodies. The high incidence of antibody was due to factors such as mild intrauterine infection of the foetuses, subclinical infections with BVDV, infections with the serologically related BDV and HCV in addition to the use of BVDV contaminated tissue culture vaccines. Because of strain

variations and the fact that in most surveys only one strain was used for the detection of antibody, it is most probable that the incidence is higher than reported (Hafez et al., 1976).

Bovine foetal calf serum had been considered as a safe supplement in tissue cultures until Kniazeff et al., (1967) observed that some FCS contained gamma-globulins which neutralized BVDV. Gillespie et al., (1967) isolated the BVDV from aborted foetuses and Braun et al., (1973) reported that prenatally infected bovine foetuses were capable of producing antibody by 200 days of gestation. Antibodies to bovine herpes-1 and parainfluenza-3 were also detected in FCS and the presence of very low titres of neutralizing antibody was found to mask the CPE of more than 10-fold of the homologous virus although the virus particles were detected by electron microscope (Swack, et al., 1975).

Infected animals from which samples are being examined may have serum neutralizing antibody and this is another difficulty. Gutekunst and Malmquist (1964) reported that they isolated BVDV from buffy coat cells even-though significant complement fixing and serum neutralizing antibody titres were present. In such cases the isolation of virus by the normal procedures which include the inoculation of whole blood, haemolysed blood or serum in tissue culture is unlikely to be successful. Virus isolation from the animal secretions and excretions should also be attempted: I successfully isolated the virus from the faeces and/or nasal

secretions of some sheep up to the 16th day P1 although they were showing some antibody titres (table 23 and Appendix table C).

Problems of Serum Contamination:

Contamination of serum with micro-organisms has always been a major concern. Animal viruses, bacterial viruses, vegetative bacteria, bacterial variants and mycoplasma have all been isolated from FCS.

Contamination with animal viruses:

As antibodies do not pass via placenta in the bovine animal the presence of BVD neutralizing antibody in 40 percent of FCS pointed a high incidence of virus infection in utero (Kniazeff et al., 1967).

Kniazeff (1968) later drew attention to the possibility that viruses like adenovirus, herpesvirus, myxovirus, picornavins, enterovirus and BVDV could be serum contaminants. Molander et al., (1969) identified infectious bovine rhinotracheitis virus, BVDV and P1-3 virus in FCS and reported that out of the 148 samples of commercial FCS they examined, 14 were contaminated with virus particles. The rate of contamination in the commercial FCS with bovine viruses ranged between 10 (Boone et al., 1972) and 75 percent (Smithies and Modderman, 1975). In the latter study all the contaminants were noncytopathic strains of BVDV, a finding which indicated the magnitude of the difficulty of a correct diagnosis of BVD infection. Hubert et al., (1973) isolated a bovine syncytial

virus from the blood of a foetal calf collected by cardiac puncture and Kniazeff et al., (1975) isolated a bovine enterovirus strain from a "sterile" commercial FCS.

Contamination with other micro-organisms:

Bacterial viruses were also reported as FCS contaminants (Merril et al., 1971; Merril et al., 1972; Chu et al., 1973; Petricciani et al., 1973; and Fong et al., 1975). The presence of bacteriophage in commercial FCS can be taken as an indication of sterility precautions and efficiency of serum preparation and storage because replication of bacteriophage depends on the availability of bacteria. Bacteriophage contamination must be considered undesirable in any in vitro culture system which utilizes FCS; the observation by Merril et al., (1971) that a phage genome was expressed in cultured human cells resulting in the change of their metabolism must alarm producers of tissue culture vaccines.

Boone et al., (1972) reported that 10 percent of the FCS they examined were contaminated with bacteria and/or fungi. Bacteria which are not sensitive to antibiotics cause turbidity and hence are easily detected. Some bacteria lose their cell wall in the presence of antibiotics and become L-forms which do not result in observable turbidity. These forms are filterable, multiply in tissue culture and are usually mistaken for debris or artifacts (Brem, 1969). They also require for their detection special media which are not normally employed in testing the sterility of sera. Such L-forms have

been isolated from apparently sterile blood (Witler et al., 1960) and human kidneys (Kalmanson and Guze, 1968). Some of the conditions which induce L-forms in vitro also exist in vivo and include serum factors, leucocytic enzymes, lysozyme, amino-acids, osmotic shock and antibiotics which interfere with cell wall synthesis and which are sometimes included in animal feeds (Brem, 1969).

The source of tissue culture contamination with mycoplasma is far from clear. Holmgren and Campbell (1960) postulated that the contamination of tissue culture with PPLO resulted from the conversion of vegetative bacteria in response to antibiotics. However, recently calf serum used for the supplementation of growth media has been suspected. Fong et al., (1975) examined some sera and reported that they isolated mycoplasma from five out of twenty five commercial bovine sera most of which were from foetuses.

ALLEVIATION OF HAZARDS

As long as serum and cell cultures are necessary for virus research and vaccines production, the threat of endogenous contamination of both media must be considered. Viruses, whether they can propagate in the cell culture or not and whether they are cytopathic or noncytopathic constitute the prime hazard. The presence of adventitious BVDV seems to be the most serious problem in both tissue and sera derived from the bovines. Improved assay methods especially for noncytopathic viruses may

reveal a considerably higher incidence of contamination.

To resolve some of the problems associated with virus contamination of cell cultures and sera, I collected lamb testes, bovine embryonic kidneys and calf testes, processed and stored them at - 114°C while I tested aliquots for adventitious viruses. In the meantime their viability was investigated.

Testing of Cell Cultures:

A high rate of contamination of BEK cells with BVDV was observed (table 2). An infection rate of 40 percent was detected when both the interference and the indirect fluorescence test were used. This finding indicated that both the interference and the fluorescence tests should be employed in testing cell cultures as only 32 percent were detected by the interference test and 24 percent by the fluorescent test. Out of the 40 percent infection rate, 20 percent were simultaneously detected by both tests. At the beginning of my studies I used to test primary cultures for virus contamination, but later I discovered that some of the kidneys showed virus in the second and third passages. I assumed that at least the third passage propagated in antibody free serum should be examined before the tissues could be considered as free from interfering adventitious BVDV.

Cryopreservation of Cells:

Cryopreservation helped to solve the contamination problems because the cells could be stored until tests for contamination were completed. Cross contamination is a serious problem in

laboratories handling noncytopathic strains of BVDV. Pre-tested reserves are therefore of prime importance.

Stored BEK cells retained their viability even when they were stored for more than a year (table 4). A drop of 46 - 54 percent in the viability of stored BEK cells was reported before (Sarma and Edwards, 1967).

My success was attributed to the protective effect of DMSO (Dougherty, 1962), but another possible explanation was the fact that BEK cells, when processed for tissue culture tended to clump. Freezing and thawing might have dissociated the aggregates to give an apparently unchanged total counts.

Results with CT cells were not as uniform. The viability of most batches did not drop significantly over 12 months. A few batches however showed about 30 percent drop. The viability of LT cells was inconsistent and showed a highly significant drop.

Monolayer formation:

The time required by the stored BEK and CT cells to form monolayers was not significantly affected by storage, but the stored LT cells required longer time to form monolayers and some aliquots failed to form monolayer on primary propagation. However on subsequent subpassages they formed good monolayers.

Propagation and Maintenance of Cultures:

The problems of inefficient cell growth, unsatisfactory monolayer formation and rapid degeneration of cell cultures

whether resulting from the BEK cells or the sera used for their propagation have been reflected by the different media used by different workers (Appendix table I). Sometimes the same worker or workers used different media for the same purpose without indicating any reason.

Seven of the media combination were used to grow BEK cells. All the media were supplemented with 10 percent FCS which was inactivated at 56°C for 30 minutes. Supplemented minimum essential medium in Earle's balanced salt solution (Rinaldo et al., 1976) gave the most consistent monolayer formation with the three levels of BEK cells seeded. EYL was also a very good medium but MEM has the advantage of being chemically defined.

Media supplemented with chemically undefined organic substances like lactalbumin hydrolysate were inconsistent. The variation in the growth supporting capacity of LHBSS was observed between two batches of the same medium ordered from the same supplier. The first batch supported the growth of BEK cells very well, but when the second batch was used for stored cells from the same kidney and with the same batch of FCS, the result was disastrous. Cells were observed to grow in small batches; they did not form monolayer even when left for longer periods. Most of the cells showed rounding and were easily detachable from the surface of the bottle by shaking. When the two batches were compared in relation to their pH, osmolarity and sodium chloride content, no significant differences were observed.

In addition to being chemically defined, MEM also had the advantage of fast monolayer formation. Heuschele, (1975) observed that when the same virus was assayed on one to two days old cultures, it usually had 0.5 to 1.0 log units higher titres than virus assayed in three days-old cultures. Better sensitivity of cultures was achieved when cells and virus dilution mixtures were inoculated at the same time especially in the microtitre neutralization test (Chapter Four). For this purpose, I propagated the second passage of cell cultures in roux flasks. When cells were fastly dividing they were resuspended in MEM with five percent BVD antibody free sheep serum.

For the maintenance of BEK, CT and LT cells MEME was found to keep the cell monolayers in good condition for five days. When it was necessary to keep the cells for longer periods two percent rabbit or sheep serum were better supplements than FCS (Fig. IV).

In areas with high incidence of BVD such as 91 percent (Borgen, 1963a) when it is difficult to get susceptible tissues and uncontaminated sera, the storage of cells is an ideal way to perform any routine or research work on BVD.

Detection of Serum Contaminants:

The system developed in these studies depended on the use of stored pretested cells for the detection of virus and antibody contamination of sera. Cells of bovine origin were considered to be the best medium for screening bovine sera because they came from the same species (McFerran et al., 1972). Even

with the availability of pretested tissue cultures, screening of bovine sera for viruses and their antibodies was not an easy job. Sera contaminated with a very small number of virus particles/or containing low titres of antibody or both could easily be missed. For example Nuttal et al., (1977) reported that viral infection of cell cultures resulting from contaminated sera was not usually detected until the sixth passage. Smithies and Modderman (1975) reported that they could detect sera with low concentration of virus by exposing BVDV negative BEK cells during their multiplication in large roller bottles to about 50 ml of the test serum because this method resulted in the replication and amplification of the small amount of virus in the serum. The problem of co-existing antibody and virus is relevant to any work with BVD. Fong et al., (1975) and Swack et al., 1975) recommended the examination of thin sections of serum pellets for the detection of virus particles.

Various workers have tried different methods to inactivate adventitious viruses in sera. I found that the treatment of serum at 56^{o}C for 30 minutes was enough to inactivate BVDV without affecting the cell growth promoting capacity of the serum for BEK, CT and LT cells. King and Harkness (1975), Nuttal et al., (1977) inactivated their sera at 60^{o}C for 30 minutes and found that the method was efficient for destroying BVDV contaminations. Fong et al., (1975) reported that the incubation of serum at 35^{o}C for 1 - 2 weeks destroyed the contaminating virus as well as mycoplasms but it

increased the possibility of bacterial contamination. The inactivation of virus in the serum by binary-ethyleneimine did not affect the growth promoting capacity of the serum (Bahnemann, 1976). However, ultraviolet light, x-ray, microwaves and B-propiolactone treatment has yet to be authenticated and their efficiency in assuring sterility and lack of destructive effect on the growth promoting capacity of the serum for different types of cells be explored.

Fogh et al., (1971) found that rabbit serum can rarely be contaminated by interfering adventitious virus or antibody. Moreover rabbits could be kept cheaply under hygienic conditions in small isolation cages. They could also be bled in sterile surroundings to avoid the possibility of extrinsic contamination of the serum. With the sterilized rabbit bleeding apparatus I used, up to 50 ml of blood were collected from each rabbit without any deleterious effects. Sheep sera sometimes have BVDV neutralizing antibody but the risk is less than with bovine sera. All the sheep bled by me during the last three years were negative to BVD antibody at 1/2 dilution of serum against 100 $TCID_{50}$ of the NADL strain of BVDV and the three commercial lamb sera lots were also negative at 1/4 dilution/ 100 $TCID_{50}$.

In the early stages of this work, all sera were tested at a 1:4 dilution against 100 $TCID_{50}$ of the NADL strain before they were used for growing cell cultures. Later, when sheep sera were used for growing BEK cells, one of the BVDV negative kidneys showed specific BVDV fluorescence which originated from kidney tissues. This led to the assumption that the FCS used

earlier for growing that particular tissue culture probably had a low titre of antibody which was not detected by the NADL strain and which masked the virus contamination.

Aliquots of some of the foetal calf sera used earlier were re-examined at 1:2 dilutions with 50 $TCID_{50}$ of the NADL strain. Although most of them had no gamma-globulins they still neutralized the virus (Appendix table 4) and the immuno-electrophoresis revealed that they all had IgG. In conclusion I would recommend that bovine sera used for tissue culture should be examined neat with varying dilutions of the virus in comparison with a known negative serum. It would also help if more than one virus strain were used.

Consistency of Serum:

For the production of sera for tissue culture with consistent chemical parameters and biological activity, some strict surveilance procedures were put forward by Boone et al., (1972) who outlined a method for strict sterility and efficient production and testing of FCS. They showed that their method resulted in the production of sera which had the recommended chemical composition when they compared their sera with commercial lots.

I ordered FCS from commercial suppliers who should have produced their sera with such strict procedures. Still some batches of serum were not successful in supporting cell growth.

With the ultimate goal of eliminating the interference and considerable variation of the serum many workers have attempted to develop serum substitutes. As yet, no serum

substitute has been reported for growing primary bovine cell cultures although the use of horse (Lee and Gillespie, 1957) and lamb sera (Gillespie et al., (1961) has been suggested.

I compared sheep and horse sera with FCS for growing BBK cells. Horse serum was very poor but sheep serum resulted in the formation of monolayers which were comparable to those formed by FCS as for the total number of cells in the monolayers. Grossly, monolayers formed by sheep serum were more uniform and healthier as they were faster in turning the medium acidic.

Serum alternatives:

It has been reported that some cell cultures can either be propagated or maintained in media without any serum supplements. Neuman and Tytell (1960), for example, compounded a serumless medium which was suitable for the propagation of primary and malignant human and monkey cells. Some cells which grew in chemically defined media with peptone supplement produced growth promoting factors for serum dependant cells (Alfred and Pumper, 1962). Jenkins and Anderson (1970) reported that 10-20 µg/ml of oleic acid replaced serum in Weymouth medium as a growth supplement for the monkey kidney cell line (LLC-MK_2). Orr et al., (1973) propagated primary monkey kidney and chicken embryos monolayers in MEM without any serum supplement. Godspodarowicz (1974) observed that a purified fibroblast growth factor (FGF) from the pituitary, when coupled with hydrocortisone stimulated DNA synthesis and cell division of 3Tc mouse cell line in a manner

comparable with serum. The FGF in combination with hydrocortisone and bovine serum albumin (BSA) completely replaced serum in the propagation of both BALB/C and 3Tc mouse cell lines (Rudland et al., 1974). Yamane et al., (1975) successfully propagated several tumour cell cultures in MEM supplemented with 1 percent BSA. Chicken kidney cell monolayers formed within five days in MEM supplemented with 0.3 percent dehydrated tryptose phosphate broth. MEM without serum was also suitable for maintaining dog kidney monolayers for 7-10 days and changing the medium was not necessary (Murakami and Kato, 1978).

PRODUCTION OF MONOSPECIFIC SERA

The availability of monospecific fluorescein-tagged antiserum can reduce the time required for the diagnosis of BVD when the right specimens are available. Confirmation of a diagnosis by fluorescence requires a few days whereas it may require a few weeks with isolation and amplification of the virus before identification can be performed by the serum neutralization and interference neutralization tests. For the complement fixation test to be of any help in diagnosis, at least five weeks should elapse after the appearance of the symptoms before a diagnostic titre could be reached (Ruckerbauer et al., 1971). The use of paired sera in the neutralization tests requires a lengthy period and sometimes it is not possible to get the second serum sample (Snowdon 1973).

Hitherto, fluorescein-conjugated BVD antisera have been unsatisfactory. Sera prepared in rabbits resulted in the fluorescence of BEK cells whether infected by BVDV or not (Fernelius and Packer, 1969). With antisera prepared in calves, the fluorescence intensity of cells infected with NCP virus and stained with fluorescein labelled NCP viral antiglobulin was higher than that of NCP virus infected cells stained with labelled cytopathic viral antiglobulin (Fernelius, 1964). Heuschele (1975) observed that there were different fluorescent intensities among the different strains of BVDV when he treated them with the same serum-fluorescein conjugate. Kniazeff et al., (1975) examined some of the fluorescein-conjugated BVD antisera used in different laboratories and reported that only one antiserum conjugate was satisfactory for detecting NCP strains of BVDV and even then the intensity of fluorescence was not of high standard.

Preparation of Antisera:

BVD antisera have usually been prepared by inoculation of animals with the virus propagated in bovine cell cultures because of the difficulty of propagating the agent in non-bovine primary cell cultures and cell lines. Most of the commercially available cell lines do not seem to be susceptible to the virus presumably because they got infected with noncytopathic BVDV strains from the sera used in their propagation. I examined RK-13, PK-15, MD-BK and vero cells by the IFT and they all showed positive fluorescence

for BVD. A trial to propagate the NADL strain into these cell lines was not successful and no CPE was produced.

Animals which have been used for the production of BVD immune sera included susceptible specific pathogen-free calves (Fernelius, 1964), gnotobiotic calves (Compton Laboratories) bovine foetuses in utero (Brown et al., 1968), rabbits (Castrucci et al., 1968; Fernelius and Packer, 1969), goats (Meyling, 1970) and sheep (Corthier and Aynaud, 1973). The use of such sera in the diagnosis of BVD led to equivocal results probably because of the non-specific reaction to bovine tissue (Fernelius and Packer, 1969) and sera (Johansson et al., 1976) in the virus antigen. Other complications arose from contamination of cell cultures and sera with adventitious virus, the immunological differences among the different strains of BVDV (Heuschele, 1975) and the poor antigenicity of BVD for fluorescent antibody studies (Kniazeff et al., 1975).

I prepared sera by hyperimmunizing rabbits with the NADL strain of BVDV propagated in BEK and LT cells. Control sera were prepared by hyperimmunizing rabbits with uninfected BEK and LT cells. Sera were also prepared in sheep and a calf.

Sera were investigated before and after adsorption with sheep liver powder. The use of bovine tissues was avoided because of the uncertainty about their content of BVDV antigen.

Gel Diffusion Reactions:

Virus and control sera prepared in rabbits formed gel

precipitin lines when diffused against infected and uninfected cells. When sera were absorbed some of the nonspecific reactions disappeared; anticellular sera did not react with the cell antigen but the viral antisera continued to react with both the viral and cellular antigens. Sera prepared in sheep and a calf did not show any gel diffusion lines.

Complement Fixation Reactions:

Both antiviral and control sera prepared in rabbits fixed complement with infected and uninfected BEK, CT and LT cells. Sheep sera were either negative or they showed weak reactions and the calf serum was negative when it was tested against the NADL strain of BVDV.

Serum Neutralization Reactions:

Both virus and control antisera prepared in rabbits neutralized BVDV in BEK and LT cells, the control antisera to a less degree. Adsorption did not remove the virus neutralizing effect of the control serum prepared with BEK cells, but it reduced the low effect in the LT cells antiserum to a barely detectable titre. Anticellular sera prepared in sheep did not neutralize the virus but antiviral sera did so.

Nonspecificity of Antisera:

These reactions showed that BVD antisera prepared in rabbits were not a useful tool in the diagnosis and identification of BVDV or the exploration of the immunological

relationship among the different strains. Castrucci et al., (1968) on the other hand reported that anti-BVDV sera prepared in rabbits had good and specific neutralization titres. Fernelius and Packer (1969) however, produced similar sera which were not specific and like my control sera, their controls neutralized the virus. They also reported that the titres of BEK cells antisera, which had as high titres as those shown by the virus antisera, varied with the different strains of BVDV and both the virus and cellular antisera formed gel precipitin lines with BVDV infected cells but not with disrupted uninfected cells.

The antiviral effects of hyperimmune anticellular sera prepared in rabbits have been reported by many investigators. Such sera have been shown to have neutralizing, complement fixing and haemagglutination inhibiting effects: Antisera to normal chicken tissue neutralized raus sarcoma virus (Amies and Carr 1939; Rubin, 1965), avian leukosis virus (Eckert et al., 1955; Beard et al., 1957) parainfluenza (Sendai) and Newcastle disease viruses (Kosyakov et al., 1966) and influenza virus (Kosyakov et al., 1966; Laver and Webster, 1966). Antisera to HeLa cells and monkey kidney cells neutralized the CPE of some strains of poliomyelitis virus, echo virus and some Coxsackie virus strains (Habel et al., 1958; Quersin-Thiry, 1958, Axler and Crowell, 1968). Antisera to foetal hamster tissues inhibited tumour formation by the simian virus-40 (SV-40) while antisera to adult hamster tissue did not (Becker et al., 1977).

Two theories have been presented for the explanation of the neutralization effect of such sera. One theory suggested that the sera contain specific antibody to the cell receptors. This antibody attaches to the virus receptor on the susceptible cell, hence blocking the portals of virus entry (Quersin-Thiry, 1958; Axler and Crowell, 1968). The alternative explanation is that, as the virus envelope is normally of cellular origin, anticellular sera has a direct effect on the virus particles because of the antigenic similarity between the virus envelope and the host cell membrane (Kosyakov et al., 1966; Laver and Webster, 1966).

My data had a similar pattern to that reported by most workers, namely that the anticellular sera showed lower titres than the antivirus sera. In that respect they did not refute either hypothesis. In contrast, Fernelius and Packer (1969) reported similar titres with virus and cellular antisera. Those high neutralization titres that fluctuated with the different strains of BVDV showed that both theories were not enough for the explanation of this phenomenon with different types of viruses. A possible explanation for this very high titre in the anticellular serum is that either the bovine foetal tissue or the bovine serum used in the cell cultures contained a low titre of undetectable NCP BVDV. The fluctuations of the titre with the different strains of BVDV favour this explanation as it was shown that specific BVD antibody titres fluctuated with different BVDV strains (Fernelius et al., 1971; Corthier and Aynaud, 1973; Castrucci et al., 1975; Hafez et al., 1976).

A second possible explanation stems from the finding that human foetal tissue (Gold and Freedman, 1965) and hamster foetal tissue (Duff and Rapp, 1970) have a common antigen with latent and oncogenic viruses such as SV-40. The fact that foetal tissues shared some antigenic properties with the SV-40 led to the successful immunization against the virus using irradiated foetal tissue (Ambrose et al., 1971, Girardi et al., 1973). This immunity was shown to be humoral because it was passively transferrable (Becker et al., 1977). Tissues with such immunological properties had to be collected at a definite stage of gestation, namely two to six months in humans (Gold and Freedman, 1965) and nine to 12 days in hamsters (Coggin et al., 1970). Immunization with 14 day gestation hamsters foetal tissue failed to induce immunity to SV-40 tumour formation (Coggin et al., 1971).

BVDV, like SV-40, has latent properties (Liess et al., 1974) and therefore it probably also shares a common antigen with the foetal bovine tissue. The very high titre of neutralizing antibody in the BEK antiserum (Fernelius and Packer, 1969) indicate that they probably collected the foetal tissues at the right stage of gestation.

Complement fixing activity has been observed between antiserum to normal chicken tissue and avian leukosis virus (Eckert et al., 1955). It has also been observed between monkey kidney cells and antisera to cell cultures of monkey kidney, human skin, human amnion, HeLa cells, mouse sarcoma, guinea pig kidney cells, culture supernatent of HeLa cells,

human serum, human red cells and rabbit serum (Habel et al., 1958). In addition the reaction has been observed between antisera to baby hamster kidney, and pig kidney cells and vesicular stomatitis virus propagated in the homologous cell cultures (Cartright and Pearce, 1968).

The sera I prepared in a calf and two sheep did not show gel diffusion reactions and although they had high titre of neutralizing antibodies, they were negative in the complement fixation test when they were tested against the NADL strain of BVDV propagated in BEK, CT and LT cells. These animals had been recently infected with the virus and they were expected to have complement fixing antibodies. Failure to demonstrate complement fixing antibodies in inactivated ruminants sera was reported before (Rice and Brooksby, 1953). Gutekunst and Malmquist (1964) apparently solved the problem by concentrating the antigen and Ruckerbauer et al., (1971) by using the indirect method originally described for ornithosis (Brumfield and Pomeroy, 1959).

Immunofluorescence:

Although the sheep serum was specific in the serum neutralization test, it showed intense nonspecific background fluorescence in infected and uninfected cultures. The serum prepared in the calf by inoculation with the NADL strain showed specific intense fluorescence with the same virus strain. Its fluorescence activity was less intense with the other strains. Challenging the calf with a NCP strain of BVDV did not improve the intensity of fluorescence with that strain

probably because the challenge was too late and by the time of challenge the antibody titre had already attained its peak.

The best serum for fluorescence studies was that prepared in the bovine animal although the intensity of fluorescence was not extremely satisfactory with all the virus strains tried and it sometimes needed careful examination to see the specific fluorescence. A possible improvement might follow the preparation of a multivalent antiserum taking into consideration the serological differences among strains of BVDV.

SUBCLINICAL BVD

A subclinical form of BVD infection was suspected by Underdahl and co-workers (1957) in the United States when they detected neutralizing antibodies to BVDV in herds with no history of the disease. A similar observation was reported in Germany (Bögel, 1964) and Australia (Snowdon, 1973). The discovery of neutralizing antibodies to BVDV in field sheep sera aroused the suspicion of subclinical form of the disease in sheep (Bögel, 1964, French and Snowdon, 1964) and the possibility of a reservoir for infecting cattle analagous to that with bovine malignant cararrhal fever (Bögel, 1966). BVDV was then isolated from a lamb in an investigation carried out to detect agents associated with ovine perinatal mortality (Hore et al., 1973).

Experimental infection of sheep with BVDV has been attempted (Ward, 1971; French et al., 1974; Snowdon et al., 1975) and it was found that the virus was infective causing a transient low grade fever in pregnant sheep (Ward 1971, French et al., 1974). Infection of pregnant sheep resulted in abortions, stillbirths, foetal autolysis, nervous disorders and muscle weakness (Ward 197 , Snowdon et al., 1975). The pathological effects on the foetus, foetal membranes and maternal caruncles varied with the virus strain (Snowdon et al., 1975). The virus was isolated from most foetal organs and foetal membranes and fluids (French et al., 1974; Snowdon et al., 1975), the highest titres of up to 10^8 $TCID_{50}$ per g. occurring in the placentomes (Snowdon et al., 1975).

Studies have concentrated on the effect of BVDV on the foetus; I likewise induced abortion in a pregnant ewe and isolated the virus from the foetus 21 days after injecting the ewe. In addition, I conducted studies on the effect of the virus on non-pregnant sheep. This included investigations of the shedding of the virus in the secretions and excretions as well as the blood of the animals inoculated by both the intranasal and intravenous routes. There was no elevation of temperature and no change in the haematological picture.

Efficiency of Virus Isolation:

Animals infected intranasally shed a low quantity of virus in their secretions and excretions and although they had a low titre of serum neutralizing antibodies the virus was isolated from samples after two or three blind passages.

but the isolation from blood was rare; only five blood samples contained virus in comparison to 16 faecal samples and 15 nasal samples. As the most probable routes of infection with BVDV in nature are the mucosae of the mouth and nostrils, secretions and excretions would appear to be better samples than blood for virus isolation from the live animal despite the difficulty of contamination with mycoplasma and antibiotic resistant bacteria.

Days eight and 12 post-inoculation were found to be the best time for virus isolation. This compares with the report of Pardo (1977) that day eight and 12 post-inoculation were the best times for BVDV isolation from infected calves.

There was also a significant difference in the overall sensitivity of BEK, CT and LT cells for virus isolation. LT cells were the most sensitive followed by CT and finally BEK cells. A possible explanation for the sensitivity of LT cells was the fact that the virus was passaged in LT cells before it was inoculated into sheep.

Sheep inoculated by the intravenous route rarely shed the virus; although a high titre was detected in their blood, only one faecal sample gave virus on day eight P1.

Virus Transmission:

The amount of virus shed by the intranasally inoculated sheep was very small, still it resulted in the seroconversion of one of the incontact sheep. Likewise BVDV was found to be transmissible from cattle continuously shedding the virus to incontact sheep as indicated by seroconversion, but it was not transmissible from intravenously inoculated sheep to

incontacts (French et al., 1974). My results were similar, but in addition, I demonstrated that intravenously inoculated sheep rarely shed the virus in their secretion and excretions. On the other hand, Plant et al., (1976) found that high titres of virus were discharged from infected aborting and lambing sheep and all their incontact controls developed appreciable titres of antibody as a result.

In addition to the amplification of BVDV by pregnant sheep, an extra hazard for cattle arises from BDV which was observed to be highly pathogenic for the bovine foetus (Gibbons et al., 1974). The virus has been shown to be prevalent in the tissues of affected lambs and aborted foetuses (Dickinson and Barlow, 1967; Shaw et al., 1967).

BVDV AND RV

The viruses of BVD, rubella and HC share some physico-chemical properties. They are all RNA viruses, ether sensitive with a lipid envelope and a diameter of less than 70 nm. Purified preparations from the different viruses observed in the electron microscope showed that their particles had similar morphological characters (Horzinek et al., 1971). This knowledge led to the approval by "The International Committee on the Taxonomy of Viruses" to group them under one family, the Togaviridae (Fenner, 1976).

A serological relationship has been reported between BVDV and HCV and trials to utilise this property in the diagnosis of BVD were successful (Darbyshire, 1960;

Mengeling et al., 1963).

Attempts to relate BVDV and RV serologically have not yet been published. The antisera I prepared to BVDV and RV failed to cross-neutralize and antisera to BVDV did not inhibit RV haemagglutinins. These results suggested that there was no immunological relationship between the two viruses.

In cell cultures, a cytopathic strain of BVDV did not show CPE in RK-13 cell line monolayers and RV propagated in cells previously inoculated with BVDV. On the other hand, BEK cells previously inoculated with RV failed to support the propagation of the cytopathic strain of BVDV. In short RV induced some form of interference with BVDV but whether this was due to particle specific interference or due to interferon production has yet to be elucidated. Unfortunately, this interference does not help in the diagnosis of BVD, but it could be used for the identification of RV strain. Interference of RV with other viruses such as myxoviruses, arboviruses, enteroviruses, papovaviruses and herpesvirus has been reported (Parkman et al., 1964)

BVDV AND BDV

In the first description of BVD, the disease was reported as being caused by a virus (Olafson et al., 1946). BD was described by Hughes and Kershaw in 1959, but Dickinson and Barlow (1967) reported that the involvement of an infectious agent in its aetiology was not suspected until 1963 when they

observed that a suffolk lamb with a hairy fleece was born to a ewe newly introduced into an experimental flock from a source where the disease had never occurred. They concluded that the agent was a virus.

Both BVDV and BDV were shown to be small viruses, sensitive to heat and ether (Hermadson and Dinter, 1962, Durham et al., 1975) and could be propagated in bovine and ovine cell cultures (Underdahl et al., 1957, Seibold and Dougherty, 1967, Hadjisavvas et al., 1975, Vantsis et al., 1976) and both viruses have CP and NCP strains. The buoyant density of BVDV strains varied from 1.09 (Hafez and Liess 1972) to 1.15 (Fernelius, 1968) with a peak for the C24V strain at a buoyant density of 1.115 (Maess and Reczko, 1970). Similar buoyant densities have been reported for BDV (Vantsis et al., 1976).

Strains from both viruses have been shown to be infective for cattle, sheep, goats and pigs, rarely causing a transient elevation of temperature in the inoculated animal (Ward et al., 1969; Gibbons et al., 1974; Ward, 1971, Plant et al., 1976 - Huck, 1973 - Stewart et al., 1971 Derbyshire, 1975). The main effect of the virus isolated from sheep on the foetuses of inoculated dams include placentitis, abortions, mummifications, stillbirth, pneumonia, diarrhoea and central nervous system involvement (Gibbons et al., 1974; Plant et al., 1976). Similar effects have been recorded for virus isolated from cattle (BVDV) on foetuses of cows and ewes (Ward et al., 1969, Casaro et al., 1971, Ward, 1971, French et al., 1974. A

mild or late infection in the third trimester may not result in serious pathological changes but only in the production of antibodies by the foetuses (Kendrick and Kronlund, 1968; French et al., 1974). Strains of the same virus vary in their ability to cause foetal death, in their degree of multiplication in the foetal tissue and the extent of pathological changes they exert on the caruncles (Snowdon et al., 1975).

The possible involvement of BVDV in the aetiology of border disease was first postulated by Acland et al.,(1972). They described a condition in sheep in Australia resembling BD which resulted in the production of gel diffusing antibody to BVDV. This finding was then substantiated by the work of Hamilton and Timony (1972, 1973)and Plant et al., (1973) who detected BVDV neutralizing antibody in sera collected from naturally and experimentally infected sheep. Huck et al., (1975) examined sera from BD infected sheep and goats and their progeny. They detected gel diffusing, complement fixing and serum neutralizing antibodies by either the NADL or the Oregon C24V strain of BVDV as antigens. They also showed that the infective agent was detected by immuno-fluorescence technique when a swine fever antiserum-fluorescein conjugate was used, a property shared by BVDV (Mengeling et al., 1963).

My studies pointed to a close serological relationship between the two viruses. Differences in the neutralization titres were small, nevertheless, the Moredun strain of BD (BDM) seemed to be more closely related to the NADL strain of

BVDV than the NCP or CT3 strains of BVDV. The results indicated a close relationship among the four strains of BVDV and BDV used for the production of antisera as well as the other strains of BVDV compared with them. The antiserum to Weybridge strain of BDV (BDW) at $^{1}/_{100}$ dilution had no neutralizing effect for the NADL strain of BVDV. Likewise the NADL antiserum had no neutralizing effect on CT3 73758 strain of BVDV although BDW antiserum reduced the titre of the virus.

These findings are of particular interest because Fernelius (1964) had suggested the division of BVDV strains into two serotypes according to their fluorescence intensity, one serotype for the cytopathic strains and the other serotype for the noncytopathic strains. In later studies, Fernelius et al., (1971) proposed the division of BVDV strains into three serotypes, one serotype for the NADL and similar strains, one serotype for the less cytopathic strains as represented by C24V and one serotype for the noncytopathic strains although there were clear serological differences among the noncytopathic strains they examined. They concluded that, "Serum-neutralization patterns obtained with the different strains of BVDV seem to indicate that each strain is serologically identifiable and though cross neutralization occurs with all known strains, a typing scheme can be constructed for epizootic and diagnostic purposes".

In these studies, similar results were observed with BVDV and BDV strains. Although every strain has a different

capacity to react with the other strains, nevertheless the relationship between BVDV and BDV is so close that they can be classified into the three serotypes suggested by Fernelius et al., (1971); one serotype should include the NADL and CT2 strains of BVDV and the moredun strain of BDV, one serotype the less cytopathic strain of BVDV (CT3) and one serotype the NCP strains of BVDV and BDW.

The immunological variations between BDV and BVDV are not very different from the immunologic variations among the different strains of BVDV and any major studies for serotyping strains of BVDV should include strains of BDV which can now be easily isolated and identified by their property of interfering with the plaque formation by CP strains of BVDV as well as by their capability of fluorescing when treated with fluorescein-conjugated HC antisera (Hadjisavvas et al., 1975, Huck et al., 1975).

CONCLUSION

These studies indicated that laboratories engaged in the diagnosis of BVD should always have virus-screened primary BEK cells, CT cells or both in store at -114°C for prompt confirmation of a provisional diagnosis. These cells survive frozen in DMSO for long periods. When screening the cells for adventitious virus one must be careful in the choice of serum supplement which should itself be free of adventitious virus and interfering antibody. Serum should be inactivated at 56°C for 30 minutes, a temperature which inactivates BVDV without affecting the growth promoting efficiency of the serum. Sera should be tested without dilution against more than one strain of BVDV.

Sheep sera are less likely to contain BVDV or BVD antibody and they were found to support the growth of BEK and LT cells. Consequently, they provide a safer supplement than the traditionally used bovine sera.

More than one test should be used on cell cultures to ensure that cells are free of adventitious virus. I used the interference and the indirect immuno-fluorescence tests and found that each test picked out different infected kidneys. Overall, the infection rate in BEK cells was 40 percent.

When FCS is used for the propagation of cell cultures, it must be prepared locally under strict precautions or bought from a reputable commercial supplier because the consistency of FCS depends on the efficiency of collection, preperation and

storage. BEK cell cultures can be maintained in MEM without serum supplement when the MEM is supplemented with 5 percent TPB.

The best samples for virus isolation were the faeces and nasal secretions collected between the eighth and twelveth days after infecting the sheep. Up to three blind passages were sometimes necessary.

For the fluorescence test, the best serum was prepared in the bovine but the intensity of fluorescence was not satisfactory. A method of improving the fluorescence intensity should be sought and a possible answer is the use of a multivalent antiserum.

In spite of the interference of rubella virus with the propagation of BVDV in cell cultures, the two viruses were immunologically unrelated because they did not cross neutralize and BVDV antisera did not inhibit the rubella virus haemagglutination. On the other hand, BVDV and BDV were serologically so similar that they could be considered as strains of BVDV.

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APPENDIX

APPENDIX TABLE (A)

DAYS REQUIRED FOR FORMATION OF MONOLAYER WITH DIFFERENT MEDIA

Medium	Average days to form monolayer	Range (Days)
199	8.80	7.18 - 10.42
MEMH	8.80	7.18 - 10.42
LHBSS	7.17	5.45 - 8.89
HYL	5.50	3.98 - 7.02
EYL	5.50	3.59 - 6.41
MEME	4.33	3.12 - 5.54

APPENDIX TABLE (B)

THE THREE GROUPS OF MEDIA ACCORDING TO THEIR EFFICIENCY IN PROMOTING BEK MONOLAYER FORMATION

Group	Media	Standard deviation
A	199 LEBSS MEMH LHBSS	2.20 $\pm$ 1.00
B	HY1, EYL	3.25 $\pm$ 0.87
C	EYL MEME	3.54 $\pm$ 0.71

APPENDIX TABLE (C)

SUMMARY OF VIRUS ISOLATIONS FROM SHEEP INOCULATED INTRANASALLY (GROUP 1) AND SHEEP INOCULATED INTRAVENOUSLY (GROUP 2) AT DAY 4, 8, 12 AND 16 POST-INOCULATION

		BLOOD SAMPLES												FAECAL SWABS												NASAL SWABS												
	Cells	BEK				LT				CT				BEK				LT				CT				BEK				LT				CT				Route of Inoculation
	Days PI	4	8	12	16	4	8	12	16	4	8	12	16	4	8	12	16	4	8	12	16	4	8	12	16	4	8	12	16	4	8	12	16	4	8	12	16	
Sheep Nos.	691	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	2	2	–	–	–	–	–	–	–	–	–	–	3	–	–	–	3	–	–	Intranasal
	676	–	–	3*	–	–	–	–	–	–	–	–	–	–	3	–	2	–	3	3	–	–	3	–	–	–	–	–	–	–	3	–	–	–	3	–	–	
	784	–	–	–	–	–	–	2	–	–	–	2	–	–	3	2	–	–	3	2	–	–	–	–	–	–	–	–	–	3	–	2	–	–	–	2	–	
	790	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	3	–	–	–	–	
	851	–	3	–	–	–	3	–	–	–	3	–	–	–	–	–	–	–	–	–	3	–	–	3	–	–	3	–	–	–	3	3	–	–	3	–	2	
	852	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	902	–	–	–	–	–	3	3	–	–	–	–	–	–	3	3	3	–	3	3	–	–	2	3	–	–	–	–	–	–	3	3	–	2	2	–	3	
	903	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	3	3	3	–	–	–	–	–	3	–	–	–	–	3	–	
Sheep Nos.	9	1	1	–	–	1	1	2	–	1	1	3	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Intravenous
	12	1	1	–	–	1	1	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	10	1	1	–	–	1	1	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	30	1	1	–	–	1	1	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	50	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	980	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	884	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	991	3	1	–	–	2	1	–	–	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	

*Numbers represent the level of passage at which virus was isolated

APPENDIX TABLE (D)

PRELIMINARY ANALYSIS OF THE DATA ON THE SHEEP INOCULATED BY THE INTRANASAL ROUTE (GROUP I)

Parameters	Number of samples	Number Positive	Chi-square	Degree of freedom	P	Assessment
Blood	96	8				
Faeces	96	25	10.809	2	< 0.010	HS
Nasal swabs	96	21				
BEK	96	11				
CT	96	18	6.701	2	< 0.050	S
LT	96	25				

HS = Highly significant differences

APPENDIX TABLE (E)

FURTHER ANALYSIS OF THE DATA ON THE EFFICIENCY OF VIRUS ISOLATION FROM THE DIFFERENT SAMPLES COLLECTED FROM SHEEP INOCULATED INTRANASALLY.

Parameters	Number of samples	Number positive	Chi-square	Degree of freedom	P	Assessment
Blood	96	8	9.367	1	< 0.010	HS
Faeces	96	25				
Blood	96	8	5.848	1	< 0.020	S
Nasal swabs	96	21				
Faeces	96	25	0.257	1	> 0.500	NS
Nasal swabs	96	21				

NS = difference not significant

S = difference significant

HS = difference highly significant

APPENDIX TABLE (F)

FURTHER ANALYSES OF THE DATA ON THE EFFICIENCY OF VIRUS ISOLATION ON THREE TYPES OF CELL CULTURES

Parameters	Number of samples	Number positive	Chi-square	Degree of freedom		Assessment
BEK	96	11	1.462	1	> 0.200	NS
CT	96	18				
BEK	96	11	5.777	1	< 0.020	S
LT	96	25				
CT	96	18	1.078	1	> 0.200	NS
LT	96	25				

NS = difference not significant

S = difference significant

APPENDIX TABLE (G)

SUMMARY OF THE ANALYSIS OF THE DATA OF VIRUS ISOLATION FROM SHEEP INOCULATED INTRANASALLY

Groups	N	Degree of freedom	Chi-square	Probability
B-F-N overall	288	2	10.809**	< 0.010 > 0.001
BEK-LT-CT-overall	288	2	6.701*	< 0.050 > 0.020
4-8-12-16 days post-inoculation (d.P.1)	288	3	27.812***	< 0.001
8-12-16 d.P.1	216	2	12.165**	< 0.010 > 0.001
4-8-12 d.P.1	216	2	20.277***	< 0.001
8-12 d.P.1	144	1	0.513	> 0.300 NS
4-16 d.P.1	144	1	1.726	> 0.100 NS
4 and 16 VS. 8 and 12 d.P.1	288	1	26.365***	< 0.001
B-F-N- 8 & 12 d.P.1	144	2	7.323*	< 0.05 > 0.020
B-F- 8 & 12 d.P.1	96	1	6.101*	< 0.020 > 0.010
F-N- 8 & 12 d.P.1	96	1	0.712	> 0.300 NS
B-N 8 & 12 d.P.1	96	1	3.554	> 0.050 NS
BEK-LT-CT-8 & 12 d.P.1	144	2	9.678**	< 0.010 > 0.001
BEK-LT 8 & 12 d.P.1	96	1	8.194**	< 0.010 > 0.001
LT-CT 8 12 d.P.1	96	1	2.843	> 0.050 NS
BEK-CT 8 & 12 d.P.1	96	1	2.122	> 0.100 NS
B-F-N 4 & 16 d.P.1	144	2	5.387*	< 0.050 > 0.020
B-F 4 & 16 d.P.1	96	1	3.376	> 0.050 NS
F-N 4 & 16 d.P.1	96	1	0.000	> 0.900 NS
B-N 4 & 16 d.P.1	96	1	3.376	> 0.050 NS
BEK-LT-CT 4 & 16 d.P.1	244	2	0.220	> 0.800 NS

B = blood; F = Faeces; N = Nasal samples
d.P.1 = day post-inoculation * = Significant;
** = Highly significant; *** = very highly significant.

APPENDIX TABLE (H)

ANTIBODY CONTENT AND THE PERCENTAGE PROTEIN FRACTION IN COMMERCIAL FCS WHICH HAD BEEN INCORPORATED IN GROWTH MEDIA

FCS No.	NAT	SNI	Percentage of the protein fraction content				
			gamma	Beta	alph 2	alpha 1	albumin
1	0.0	0.5	0	5	4	35	56
2	0.4	1.8	0	9	2	39	50
3	0.9	2.2	0	10	2	40	48
4	0.3	1.8	0	11	4	35	50
5	0.3	1.3	0	7	3	39	51
6	1.0	2.2	6	11	0	40	43
7	0.0	0.0	0	6	2	37	55

NAT = neutralizing antibody titre to 50 $TCID_{50}$ of CT3 73758 strain

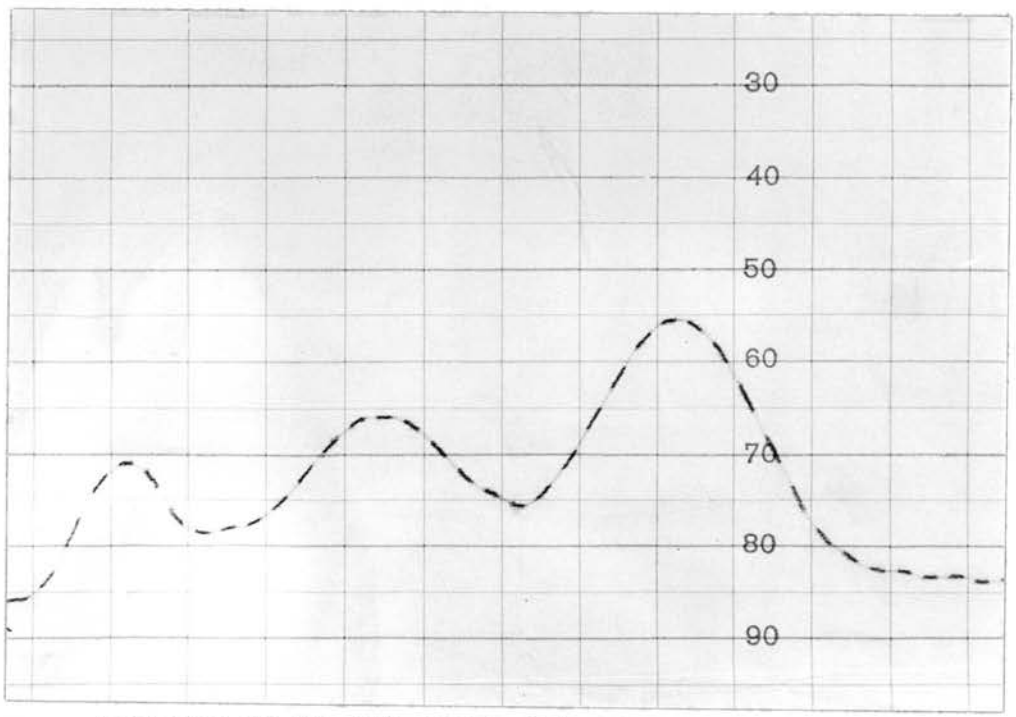
SN1 = neutralization indices to the NADL strain

APPENDIX TABLE (I)

MEDIA USED FOR GROWING BEK CELLS BY DIFFERENT WORKERS

Constituents	Reference
HBSS, 0.5 percent LAH, 20 percent bovine serum (BS)	Underdahl et al., 1957
EBSS, 0.5 percent LAH, 10 percent HS	Lee and Gillespie, 1957
Morgan, Morton and Packer 199 medium	Noice and Schipper, 1959
HBSS, 0.5 percent LAH, 0.1 percent YE, 10 percent BS	French and Snowdon, 1964
EBSS, 0.5 percent LAH, 0.1 percent bovine albumin fraction V, 0.1 percent YE, 10 percent FCS	Gillespie et al., 1967
Basal medium of Eagle, 10 percent calf serum	Ruckerbauer et al., 1971
MEME, MEM vitamins, NEA, FCS	Rinaldo et al., 1976
Eagle's medium, LAH, YE, peptone, 10 percent FCS	Rossi and Kiessel, 1971

APPENDIX FIG. 1



ELUTION CURVE FOR BVDV SUSPENSION IN MEME SUPPLEMENTED WITH 2 PERCENT SHEEP SERUM AND CONCENTRATED 50X

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